Studies on the metabolism of pantothenic acid in microorganisms(Dissertation_全文)

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Abbreviations: CoA, coenzyme A (CoASH and CoASSCoA indicate reduced CoA and oxidized CoA, respectively.); 3'-dephospho-CoA, 3'-dephospho-coenzyme A; ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; 3',5'-ADP, 3'-phosphoadenosine 5'-monophosphate; AMP, adenosine 5'-monophosphate; GTP, guanosine 5'-triphosphate; GDP, guanosine 5'-diphosphate; GMP, guanosine 5'-monophosphate; ITP, inosine 5'-triphosphate; IMP, inosine 5'-monophosphate; CTP, cytidine 5'-triphosphate; CDP, cytidine 5'-diphosphate; CMP, cytidine 5'-monophosphate; UTP, uridine 5'-triphosphate; UDP, uridine 5'-diphosphate; UMP, uridine 5'-monophosphate; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; FAD, flavin adenine dinucleotide; EDTA, disodium ethylenediamine tetraacetate; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; DEAE-cellulose, diethylaminoethyl cellulose.

INTRODUCTION

Ten years elapsed between the time, 1936, that pantothenic acid was first found to participate in carbohydrate metabolism (1) and the time that the mechanism of its function was established. The realization that pantothenic acid itself is not the functional form of the vitamin began with the discoveries that a cofactor was involved in the acetylation of choline in extracts of rabbit brain (2,3) and that of sulfanilamide in pigeon liver (3,4), and that this cofactor, named "coenzyme A" by Lipmann in 1946 (3), contained a "bound" pantothenic acid (5). A turning point in the physiological and structural studies on CoA was the discovery by Lynen *et al.* (6,7) in 1951 that "active acetate" is an acyl mercaptan. About ten years later, another turning point in the studies relating to CoA was brought from the discovery that 4'-phosphopantetheine, which also contains a "bound" pantothenic acid as well as CoA, functions in fatty acid synthesis as a protein bound prosthetic group (8). The numerous roles played by these two coenzymes derived from pantothenic acid in intermediary metabolism have been the subject of intensive research. It has now been evident that CoA is necessary not only for the activation of acetate but also for the activation of many other carboxylic acids and for the transacylation in various biological systems, and 4'-phosphopantetheine is necessary not only for the elongation of fatty acids but also for the synthesis of peptides (9).

As to the biosynthesis of CoA from pantothenic acid, the first evidence that cysteine serves as the precursor of the sulfur-containing fragment of CoA was supplied by Pierpoint and Hughes (10). They reported that CoA synthesis from pantothenic acid by whole cells of *Lactobacillus arabinosus* is dependent on the presence of cysteine. This finding was followed by the observation of Brown and Snell (11) that *Proteus morganii* also forms CoA in the presence of cysteine. In 1954, Levintow and Novelli (12) and Hoagland and Novelli (13) proposed a pathway for the biosynthesis of CoA in mammalian systems, in which pantothenic acid couples with cysteine to yield pantothenoylcysteine as the first step, followed by decarboxylation to pantetheine, which is then phosphorylated to form 4'-phosphopantetheine, and they suggested that the pathway is as follows: pantothenic acid $\longrightarrow$ pantothenoylcysteine $\longrightarrow$ pantetheine $\longrightarrow$ 4'-phosphopantetheine $\longrightarrow$ 3'-dephospho-CoA $\longrightarrow$ CoA. In 1959, however, Brown (14) proposed an alternative pathway using rat liver, rat kidney, and *Proteus morganii*, in which pantothenic acid is first phosphorylated to yield 4'-phosphopantothenic acid by the enzyme pantothenate kinase prior to coupling with cysteine. 4'-Phosphopantothenic acid is then condensed with cysteine in the presence of an enzyme designated "coupling enzyme" (4'-phosphopantothenoylcysteine synthetase) to yield 4'-phosphopantothenoylcysteine, which in turn is decarboxylated to 4'-phosphopantetheine by the enzyme 4'-phosphopantothenoylcysteine decarboxylase. 4'-Phosphopantetheine is then converted to CoA with ATP by the enzymes 3'-dephospho-CoA pyrophosphorylase and 3'-dephospho-CoA kinase. The sequence is summarized as follows: pantothenic acid $\longrightarrow$ 4'-phosphopantothenic acid $\longrightarrow$ 4'-phosphopantothenoylcysteine $\longrightarrow$ 4'-phosphopantetheine $\longrightarrow$ 3'-dephospho-CoA $\longrightarrow$ CoA. In these processes 4 moles of ATP are required for one mole of CoA. Brown (14) also suggested that in both mammalian and microbial systems this is the only operative pathway. Later, Abiko (15,16) and Abiko *et al.* (17) revalued these two pathways in detail, and confirmed that Brown's pathway operates in rat liver. However, the pathway envisaged by Novelli may function in one bacterium, *Lactobacillus helveticus*. Evidence for this includes (a) pantothenoylcysteine decarboxylase is present (14); (b) this organism when supplied with large amounts of pantothenic acid will not accumulate 4'-phosphopantothenic acid (18); (c) mutants of *Lactobacillus helveticus* can

use pantothenoylcysteine as a growth factor (19). Two other organisms, *Lactobacillus bulgaricus* and *Acetobacter suboxydans*, which can also utilize pantothenoylcysteine, possibly also follow Novelli's pathway (19).

The first highly purified preparations of CoA were obtained by extracting it from *Streptomyces fradiae* (20), and a series of its selective enzymatic digestions showed that it contains three equivalents of phosphate and each one equivalent of adenosine and sulfur for each mole of pantothenic acid (21), and can be accounted for as a pyrophosphate ester that links the adenosine 3'-monophosphate through its 5'-position to the 4'-position of pantetheine (7,22-24). During this same period, isolation procedures for CoA also had been improved by contributions by Beinert *et al.* (25) and by Stadtman and Kornberg (26). Beinert *et al.* (25) obtained highly pure CoA from baker's yeast by coprecipitation with cupper-glutathion. This was later improved by Reece *et al.* (27). On the other hand, the structure of CoA established on the basis of degradation data was first confirmed by a total synthesis accomplished by Moffatt and Khorana (28) in 1959. CoA has been supplied only by extraction from microorganisms (20,21,25-27,29) and chemical synthesis (28,30-32). However, these methods are not practical because of their lower yield or their intricacy. The unsufficient supply of CoA may give one of the disadvantages in experiments of biochemistry and clinical medicine.

Developments of seasoning manufacture, especially in Japan, have now permitted easy supply of a variety of nucleosides and nucleotides. By using them, many investigations on synthesis of more complex nucleotides, such as nucleoside triphosphates, sugar nucleotides, nucleotide coenzymes, and so on, have been done intensively (33). Tochikura *et al.* (34) have reported an efficient synthesis of ATP from adenosine or AMP during the fermentation of yeasts. It is interesting, therefore, to synthesize CoA through the supply of ATP under such fermentative conditions.

The compounds which have a close structural similarity with naturally occurring vitamins are generally known to possess inhibitory effects on growth of certain organisms or on action of certain enzymes. Some of these compounds have proven of interest in biochemical studies and in chemotherapy (35). Pantothenyl alcohol, an alcohol analog corresponding to pantothenic acid, has been reported to be converted by warm-blooded animals into pantothenic acid and to serve as an available source of the vitamin for these organisms (36-38), while the analog not only is not utilized in place of the vitamin by lactic acid bacteria, but on the contrary it prevents competitively the utilization of pantothenic acid by these organisms (39,40). However, little is known of the metabolism of this alcohol in other microorganisms. It seems, therefore, to be interesting to know the microbial response of this antimetabolite.

In this thesis the author describes the microbial metabolism of pantothenic acid and its related compounds. In the first chapter, searching the ability of the formation of CoA from pantothenic acid and cysteine under the coupling with ATP-generating system of various microorganisms, and accumulation of CoA and its biosynthetic intermediates, then on the basis of the results of these investigations a new process for the production of CoA are described. The mechanism of the accumulation of these compounds is also discussed. In the second chapter, some aspects of degradation metabolism of pantothenyl alcohol are briefly described.

CHAPTER I.

SYNTHESIS OF COENZYME A BY MICROORGANISMS

Section I.

Distribution of Coenzyme A Accumulating Activity in Microorganisms and Isolation of Reaction Products[a,b]

The ability of the formation of CoA from pantothenic acid and cysteine in the presence of AMP or ATP was searched in yeasts and bacteria. The result of screening showed that the activity was found in several yeasts and bacteria belonging to the genera *Sarcina*, *Corynebacterium*, and *Brevibacterium*. Particularly, *Brevibacterium ammoniagenes* IFO 12071 accumulated a large amount of CoA. Isolation of the reaction products, which were synthesized by *Brevibacterium ammoniagenes* IFO 12071, were carried out. The isolates were identified as CoA, 3'-dephospho-CoA, and 4'-phosphopantothenic acid.

INTRODUCTION

The numerous roles that CoA plays in intermediary metabolism have been discussed by many investigators, since the discovery of this compound as a requirement for acetylation of choline and aromatic amines by Lipmann (3) in 1946.

CoA has been prepared by extraction from microorganisms (20,21,25-27,29) and chemical synthesis (28,30-32). However, these methods are not available because of their lower yield or their intricacy.

Although studies on the biological conversion of biosynthetic precursors including pantothenic acid into CoA have been reported *in vivo* and *in vitro* (41), little is known of accumulation of CoA by microbial process. Only it was reported that a relatively large amount of CoA was synthesized by the washed cell suspension of *Lactobacillus arabinosus* which was grown in pantothenate-deficient medium (10). As demonstrated by Brown (14), four moles of ATP are required during the synthesis of one mole of CoA from pantothenic acid. Tochikura *et al.* (34) have reported that adenosine or AMP is phosphorylated to ATP under the fermentation process of yeast in the presence of a high concentration of inorganic phosphorus. It is interesting, therefore, to synthesize CoA through the supply of ATP under such fermentative conditions. In this section, the result of screening of CoA accumulation by various microorganisms and isolation of CoA and its related compounds are described.

MATERIALS AND METHODS

Microorganisms and cultivations. Baker's yeast was obtained from Oriental Yeast Co., Ltd., Tokyo. Other microorganisms used were the strains preserved in the Laboratory of Applied Microbiology, Department of Agricultural Chemistry, Kyoto University. Yeasts were grown in a medium containing 5 g of glucose, 0.5 g of peptone, 0.1 g of yeast extract, 0.2 g of KH_2PO_4, 0.1 g of K_2HPO_4, and 0.02 g of $MgSO_4 \cdot 7H_2O$ in 100 ml of tap water, pH 6.5. Bacteria were grown in a medium containing 1 g of glucose, 1.5 g of peptone, 0.3 g of K_2HPO_4, 0.2 g of NaCl, and 0.02 g of $MgSO_4 \cdot 7H_2O$ in 100 ml of tap water, pH 7.0. Each culture was carried out with 500 ml medium placed in 2 liter shaking flask under reciprocal shaking at 28°C for 2-4 days.

Preparation of dried cells. The cells harvested by centrifugation were washed with 0.85% NaCl. The paste of the cells was dried with an electric fan at room temperature, and then put in a desiccator over P_2O_5 *in vacuo* at 4°C. The dried cells were stored at -15°C before used.

Chemicals. CoA was kindly provided by Dr. E. Ohmura, Takeda Chemical Industries Co., Ltd., Osaka, and also obtained from Sigma Chemical Co., St. Louis. 3'-Dephospho-CoA and 4'-phosphopantothenic acid were kindly gifted by Dr. M. Shimizu, Daiichi Seiyaku Co., Ltd., Tokyo. ATP was kindly gifted by Dr. K. Tanaka, Kyowa Hakko Kogyo Co., Ltd., Tokyo. DEAE-cellulose (0.9 meq/g) was provided from Green Cross Corporation, Osaka. Phosphotransacetylase of *Clostridium kluyveri* was purchased from Boehringer, Mannheim. Phosphodiesterase of *Crotalus adamanteus* and alkaline phosphatase from calf intestinal mucosa were from Sigma Chemical Co., St. Louis. Acetylphosphate was synthesized according to the method of Avison (42). All other reagents were commercial products of analytical grade of purity.

Screening method. For the screening of CoA accumulation, the following standard condition was used: The reaction mixture which contained 10 μmoles of sodium pantothenate, 10 μmoles of cysteine, 15 μmoles of AMP, 167 μmoles of glucose, 200 μmoles of potassium phosphate buffer, pH 7.0, and 100 mg of dried cells was incubated at 37°C in a total volume of 1 ml. For the reaction in bacterial system, equimolar amounts of ATP were also added instead of AMP. For the screening of pantothenate kinase activity in bacterial cell system, the following condition was used: The reaction mixture containing, in 1 ml, 2 μmoles of sodium pantothenate, 10 μmoles of ATP, 150 μmoles of potassium phosphate buffer, pH 6.0, 10 μmoles of $MgSO_4$, and 100 mg of dried cells was incubated at 37°C for 2 hr. The reactions were stopped by immersing the tubes in boiling water for 5 min. The products were assayed after the removal of cells by centrifugation.

Analytical methods. CoA assays were mainly performed by the phosphotransacetylase method of Stadtman *et al.* (43) using the enzyme of *Escherichia coli* Crookes AKU 0001 and also by the method of Bergmeyer *et al.* (44) for characterization of isolated products. Pantothenic acid was measured microbiologically using *Lactobacillus plantarum* ATCC 8014 (45) and *Saccharomyces carlsbergensis* ATCC 9080 (46) as test organisms. Bound pantothenates were assayed by the method of Novelli (47). Adenine content was determined by ultraviolet absorption in 0.1 M HCl using E_{260} = 15,000 as a standard value. Phosphorus was measured by the method of Fiske-Subbarow (48), and sulfhydryl by the method of Ellman (49). Paper chromatography by the ascending technique was performed on Toyo Roshi No.53 paper using the following solvents: solvent I, isobutyric acid-0.5 N ammonium hydroxide-0.1 M EDTA (100:60:1.6, by vol.); solvent II, ethanol-1 M ammonium acetate, pH 7.5 (5:2, by vol.); solvent III, ethanol-0.5 M ammonium acetate buffer, pH 3.8 (5:2, by vol.); solvent IV, *n*-butanol-acetic acid-water (5:2:3, by vol.); solvent V, *n*-propanol-28% ammonium hydroxide-water (6:3:1, by vol.). Paper electrophoresis was performed on Toyo Roshi No.53 paper impregnated with 0.05 M sodium acetate buffer, pH 5.1, at 40 mA per 15 cm (4 KV) for 30 min. Adenine derivatives were located on the chromatogram with a UV-lamp. Pantothenic acid and its phosphorylated compound were detected by bioautographic techniques. Phosphorus-containing compounds were located with Hanes and Isherwood spray (50) followed by ultraviolet irradiation, and sulfhydryl or disulfide compounds with Toennies and Kolb spray (51). β-Alanine was detected by ninhydrin spray.

Preparation of phosphotransacetylase from Escherichia coli Crookes AKU 0001. The washed cells (12 g, dry weight) were suspended in 100 ml of 0.1 M Tris-HCl buffer, pH 7.4, and treated with a Kaijo-Denki 19 kHz ultrasonic oscillator for 10 min. The intact cells and *debris* were removed by centrifugation

at 12,000 x *g* for 20 min. To the supernatant, solid ammonium sulfate was added to give 30 to 40% saturation. The precipitated protein was collected by centrifugation at 12,000 x *g* for 20 min and was dissolved in 10 ml of the same buffer containing 10 mg of reduced glutathione amd 10 mg of bovine serum albumin. All the operations were carried out below 4°C. This protein solution was used as the phosphotransacetylase preparation.

RESULTS

Distribution of CoA-accumulating activity in microorganisms

As shown in Table I, the activity of the accumulation of CoA from pantothenic acid, cysteine, and AMP was found in some strains of yeasts, *e.g.* baker's yeast, *Saccharomyces sake* AKU 4022, *Saccharomyces lactis* IFO 1090, beer yeast AKU 4037, *Torulopsis candida* IFO 0768, *Schizosaccharomyces liquefaciens* IFO 0358, and so on. Under the conditions employed, all of them showed strong AMP-phosphorylating activity. However, inspite of their strong activity to phosphorylate AMP, some yeasts, such as *Hansenula beijerinckii* IFO 0981, *Candida utilis* IFO 0396, and *Brettanomyces claussenii* IFO 0627, did not show the accumulation of CoA. It was also observed that yeasts degrading AMP to adenosine, inosine, adenine, or hypoxanthine did not show the accumulation of CoA. While, neither consumption of pantothenic acid nor accumulation of CoA was observed in a similar reaction system with bacteria. When ATP was added to the reaction mixture in place of AMP, the consumption of pantothenic acid in the reaction mixture was obser-

TABLE I. ACCUMULATION OF CoA BY YEASTS

The reactions were carried out for 5 hr (*3 hr) under the conditions as described in the text.

Strain	CoA found (μg/ml) before reaction	CoA found (μg/ml) after reaction	Metabolites of AMP
Baker's yeast	23	128	ATP, ADP
Distillery yeast M AKU 4006	45	73	"
Beer yeast AKU 4037	58	100*	"
Schizosaccharomyces liquefaciens IFO 0358	30	80	"
Saccharomyces sake Kyokai No. 2	31	65	"
" " AKU 4017	68	86*	"
" " AKU 4022	43	85*	"
" *lactis* IFO 1090	33	75	"
" *rosei* IFO 0252	39	20	"
" *chevalieri* IFO 0210	64	56	"
" *carlsbergensis* IFO 0461	15	6	adenosine
Candida mycoderma IFO 0164	26	64*	ATP, ADP
" *utilis* IFO 0396	15	22	"
Torulopsis candida IFO 0768	28	77	"
" *sake* IFO 0435	18	75	"
" *globosa* IFO 0953	51	26	"
Hansenula jadinii IFO 0987	24	16	"
" *beijerinkii* IFO 0981	15	22	"
" *matritensis* IFO 0945	34	21	hypoxanthine
" *anomala* AKU 4300	39	43	"
Rhodotorula rubra IFO 0890	42	28*	adenine, adenosine
" *marina* IFO 0879	30	3	"
Brettanomyces claussenii IFO 0627	41	16	ATP, ADP
Saccharomycodes ludwigii IFO 1043	57	13	AMP
Lipomyces starkeyi IFO 0678	0	6	"
Trigonopsis variabilis IFO 0671	37	31	"
Schwanniomyces occidentalis IFO 0371	30	6	adenine
Debariomyces subglobosus IFO 0794	17	22*	adenosine
Kloekera africana IFO 0633	30	33*	"

TABLE II. ACCUMULATION OF CoA BY BACTERIA

The reactions for CoA formation were carried out for 6 hr under the conditions as described in the text. The mixture omitting sodium pantothenate and cysteine was used as a control run. The values of each control run are given in parentheses. Assay condition for pantothenate kinase activity is given in the text, and the values obtained are given as per cent of pantothenic acid consumed. The mixture omitting ATP was used as a control run. The values of each control run are given in parentheses.

Strain	CoA found (μg/ml) before reaction	CoA found (μg/ml) after reaction	Pantothenate kinase activity	Metabolites of ATP
Escherichia coli B AKU 0012	21	11 (5)	0 (0)	AMP, adenine
" *freundii* S-96 AKU 0009	30	18 - a)	0 (0)	adenine
Aerobacter aerogenes IFO 3320	48	18 (37)	20 (0)	AMP
Erwinia aroideae IFO 3830	63	45 (53)	20 (0)	AMP, adenosine
Serratia marcescens IFO 3046	66	58 (44)	15 (0)	AMP
Proteus morganii IFO 3838	12	18 (12)	0 (0)	AMP, adenosine
Alcaligenes faecalis IAM B-141-1	45	91 (91)	0 (0)	AMP
Achromobacter aceris IFO 3166	30	17 -	0 (0)	-
Flavobacterium fuscum AKU 0140	14	15 (12)	0 (0)	hypoxanthine
Bacillus subtilis IFO 3007	30	25 -	0 (0)	AMP, adenosine
Agrobacterium tumefaciens IAM B-26-1	25	23 (24)	0 (0)	adenine
Micrococcus luteus IFO 3763	40	57 (25)	15 (5)	AMP
" sp. No. 431 AKU 0511	56	70 (81)	0 (0)	hypoxanthine
Staphylococcus aureus IFO 3060	25	26 -	- -	-
Sarcina aurantiaca IFO 3064	58	98 (35)	55 (10)	AMP
" *lutea* IFO 1099	68	171 (63)	75 (15)	"
" " IFO 3232	64	181 (67)	70 (5)	"
" *variabilis* IFO 3067	72	89 (97)	65 (5)	AMP, ADP, ATP
Corynebacterium glutamicum ATCC 13032	61	53 (62)	80 (25)	AMP, ADP
" " ATCC 13059	60	101 (51)	80 (5)	AMP
" " ATCC 13060	42	108 (40)	70 (20)	"
" *equi* IAM 1038	50	60 (61)	25 (10)	"
Arthrobacter simplex IFO 3530	39	60 -	40 (0)	"
Brevibacterium divaricatum 1627 NRRL 2311	123	98 (85)	70 (5)	AMP, ADP
" *ammoniagenes* IFO 12071	19	830 (0)	100 (5)	"
" " IFO 12072	16	189 (33)	85 (10)	"
" sp. P145 AKU 0643	125	115 (122)	0 (5)	"
" sp. AKU 0644	109	114 (107)	60 (5)	"
" sp. AKU 0645	90	124 (87)	60 (0)	"
Bacterium cadaveris IFO 3731	62	18 (12)	0 (0)	hypoxanthine
Pseudomonas fluorescens IFO 3461	55	44 (35)	0 (5)	AMP

a) not tested.

ved by several strains belonging to the genera *Sarcina*, *Corynebacterium*, and *Brevibacterium* (Table II). Among them *Brevibacterium ammoniagenes* IFO 12071 showed a remarkable accumulation of CoA. A typical time course for CoA synthesis by *Brevibacterium ammoniagenes* IFO 12071 is shown in Fig. 1. The amounts of CoA synthesized from pantothenic acid and from pantethine after 8 hr incubation reached about 1.3 μmoles (1.0 mg) per ml and 1.7 μmoles (1.3 mg) per ml, respectively. The omission of these pantothenate derivatives from the reaction mixture gave no accumulation of CoA. An addition of sodium laurylbenzenesulfonate brought an increase of the amount of CoA synthesized. When cysteine was omitted from the reaction mixture, more than 95% of pantothenic acid added disappeared after 8 hr incubation without the accumulation of CoA. The disappearance of pantothenic acid was completely recovered by the treatment with alkaline phosphatase, suggesting the presence of conjugated forms of pantothenic acid in the reaction mixture.

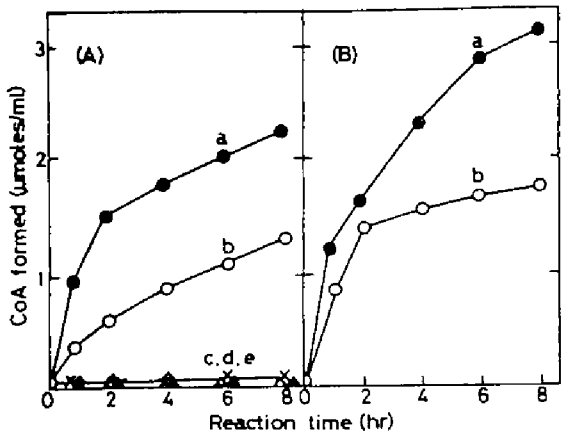

FIG. 1. Time Course for CoA Synthesis.

(A) Synthesis from pantothenic acid: The reaction mixture containing, in 1 ml, 5 μmoles of sodium pantothenate, 10 μmoles of cysteine, 15 μmoles of ATP, 10 μmoles of $MgSO_4$, 150 μmoles of potassium phosphate buffer, pH 6.0, and 100 mg of dried cells of *Brevibacterium ammoniagenes* IFO 12071 was incubated at 37°C with (a) or without (b) 2 mg of sodium laurylbenzenesulfonate. The mixture without sodium pantothenate (c) or cysteine (d and e) was used as a control run. (The mixture (e) contained 2 mg of sodium laurylbenzenesulfonate.)

(B) Synthesis from pantethine: The reaction conditions were the same as those in (A) except for 2.5 μmoles of pantethine replacing sodium pantothenate.

Isolation and characterization of the reaction products

Isolation of the reaction products. The reaction mixture containing 3 mmoles of sodium pantothenate, 6 mmoles of cysteine, 6 mmoles of ATP, 3 mmoles of $MgSO_4$, 45 mmoles of potassium phosphate buffer, pH 6.0, 600 mg of sodium laurylbenzenesulfonate, and 30 g of dried cells of *Brevibacterium ammoniagenes* IFO 12071 in a total volume of 300 ml was incubated for 10 hr at 37°C. Then, the mixture was immersed for 10 min in a boiling water and cells were removed by centrifugation. The supernatant was placed onto an active charcoal column (5.5 x 10 cm) after adjusting to pH 3.0 with diluted HCl. The column was washed with water and the adsorbed materials were eluted with ethanol-ammonium hydroxide-water (50:5:45, by vol.). The eluate was concentrated to about 100 ml under reduced pressure below 35°C. After adjusting to pH 7.0 with ammonium hydroxide, 25 ml of 2-mercaptoethanol were added to the concentrate and the mixture was left overnight at 7°C, after which it was applied to a column of DEAE-cellulose. The elution was carried out as shown in Fig. 2. Each

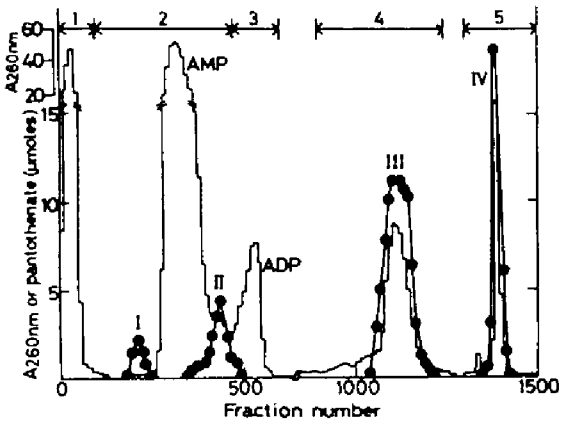

FIG. 2. A Typical Elution Pattern of the Reaction Products from a DEAE-cellulose Column.

Products were eluted from the column (chroride form, 5.5 x 36 cm) with 0.003 M HCl (1), 0.016 M LiCl in 0.003 M HCl (2), 0.02 M LiCl in 0.003 M HCl (3), 0.05 M LiCl in 0.003 M HCl (4), and 0.2 M LiCl in 0.003 M HCl (5). Each fraction contains 18 ml of eluate. Peak I, free pantothenic acid; Peak III, CoASH; Peak IV, CoASSCoA; Peak II contains mainly 4'-phosphopantothenic acid and 3'-dephospho-CoA. —— , absorbance at 260 nm; —●— , pantothenate.

pantothenate-containing peak was pooled, adjusted to pH 4.5 with diluted LiOH, and evaporated under reduced pressure below 35°C. To the residue, a small volume of methanol and 20 volumes of acetone were added to give a white powder. After removing LiCl by repeated extractions with methanol-acetone (1:15, by vol.), it was dried over P_2O_5 *in vacuo* to yield the lithium salts of pantothenate-containing compounds.

Characterization of the isolates.
i) CoASH. The isolated sample (Peak III), 391 mg, was well identical with authentic CoASH in high voltage paper electrophoresis and paper chromatography (Table III). It gave pantothenic acid by the procedure of Novelli (47), and 3',5'-ADP and a sulfhydryl compound

TABLE III. PAPER CHROMATOGRAPHY AND PAPER ELECTROPHORESIS

Compound	*Rf* in solvent					Mobility[a]	
	I	II	III	IV	V	A	B
CoASH							
authentic	0·57	0·15	0·32	0·24	–[b]	15·4	14·5
isolated	0·57	0·15	0·31	0·24	–	15·4	–
CoASSCoA							
authentic	0·40	0·01	0·10	–	–	17·1	–
isolated	0·39	0·01	0·11	–	–	17·1	–
DP-CoASH							
authentic	0·69	0·41	0·42	–	–	10·7	–
isolated	0·69	0·41	0·40	–	–	10·7	–
P-PaA							
authentic	0·53	–	–	0·59	0·33	–	16·4
isolated	0·53	–	–	0·59	0·34	–	16·5
AMP	0·56	0·13	0·29	0·28	0·21	10·4	8·4
ADP	0·45	0·03	0·20	0·19	0·19	–	–
3',5'-ADP	0·44	0·03	0·19	–	–	–	–
ATP	0·37	0·02	0·15	0·13	0·15	–	–
PaA	0·73	–	–	0·79	0·83	–	9·5
Pantethine	0·84	–	–	0·83	0·85	–	–
Cysteine	0·60	–	–	0·48	0·48	–	–
β-Alanine	0·63	–	–	0·50	0·61	–	–

a) Migration towards the anode is given in cm.
b) not examined.
Abbreviations used: DP-CoASH, 3'-dephospho-CoASH; P-PaA, 4'-phosphopantothenic acid; PaA, pantothenic acid.

(*Rf*, 0.65 in solvent I) by hydrolysis with *Crotalus adamanteus* phosphodiesterase. The latter substance is presumably 4'-phosphopantetheine. The purity was calculated to be 87% by the method of Bergmeyer *et al.* (44). The ultraviolet absorption spectrum was characteristic of an adenine-containing compound (UV λmax in 0.1 M HCl, 259 nm; UV λmax in 0.1 M NaOH, 260 nm). IR spectrum was as follows: IR νmax in KBr pellet, 723,800,825,870,955,1085,1125,1245, 1335,1371,1424,1480,1550,1655,1697,2970, 3330 cm^{-1}. Elemental analysis showed C,27.04; H,5.05; N,10.42%. $C_{21}H_{33}O_{16}N_7P_3SLi_3 \cdot 8H_2O$ requires C,27.13; H,5.37; N,10.54%. Ratio of adenosine : pantothenic acid : sulfhydryl : phosphorus, 1:0.92:0.92:2.94; required, 1:1:1:3.

ii) CoASSCoA. The yield of Peak IV was 153 mg. The purity was calculated to be 89% from the pantothenate content. The isolate showed no activity to phosphotransacetylase of *Clostridium kluyveri* (Fig. 3). Ratio of adenosine : pantothenic acid : phosphorus was 1:0.92

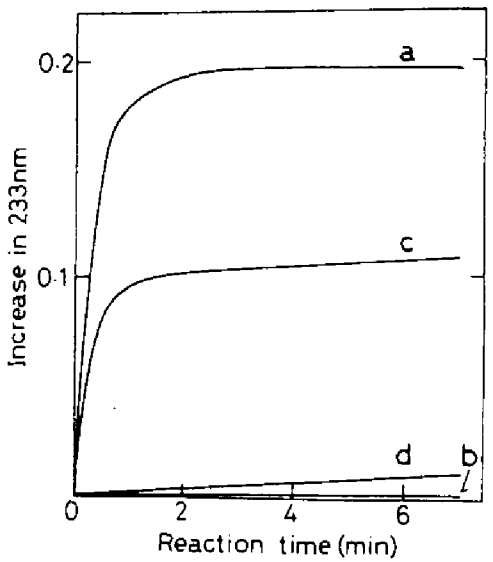

FIG. 3. Acetyl CoA Formation by Phosphotransacetylase.

Each 1 cm cuvette contained 260 μmoles of Tris-HCl buffer, pH 7.4, 26 μmoles of acetyl phosphate, 30 μmoles of $(NH_4)_2SO_4$, and 3.3 μg of the enzyme (100 units/mg) in a total volume of 3 ml with 0.13 μmole of the isolated CoASH (a); 0.13 μmole of the isolated CoASSCoA (b); both 0.07 μmole of the isolated CoASH and the isolated 3'-dephospho-CoASH (c); or 0.6 μmole of the isolated 3'-dephospho-CoASH (d).

:2.88; reqiured, 1:1:3. However, after the reduction with 2-mercaptoethanol, it was shown chromatographically to contain reduced CoA and a little of the disulfide. Assayed the reduced sample by the method of Bergmeyer *et al.* (44), it now showed 83% activity. Ratio of adenosine : pantothenic acid : sulfhydryl : phosphorus was 1:0.94:0.86:2.91; required, 1:1:1:3. Other characteristics are shown in Table III.

iii) 4'-Phosphopantothenic acid and 3'-dephospho-CoASH. Peak II was further purified by DEAE-cellulose column chromatography using a 2 liter linear salt gradient (0–0.05 M LiCl in 0.003 M HCl; column size, 2.6 x 65 cm; flow rate, 1 ml/min). Two major pantothenate-containing peaks were separated. The first eluate (after AMP) was worked up as described above to give a white powder. It contained phosphorus and neither sulfhydryl nor disulfide. Behaviors on paper chromatography and paper electro-

phoresis were almost identical with synthetic 4'-phosphopantothenic acid (Table III). This compound supported, partially, the growth of *Lactobacillus plantarum* ATCC 8014 and that of *Saccharomyces carlsbergensis* ATCC 9080. Free pantothenic acid was liberated from the isolate by the hydrolysis with alkaline phosphatase. From these results, the isolate was identified as 4'-phosphopantothenic acid. The yield was 135 mg. The purity based on pantothenic acid was 62%. Another eluate was obtained as a white powder with the yield of 28 mg. It showed about 0.8% of CoA activity to phosphotransacetylase of *Clostridium kluyveri* under the conditions employed (Fig. 3). Hydrolysis of the isolated sample with *Crotalus adamanteus* phosphodiesterase gave AMP and a sulfhydryl compound (*Rf*, 0.65 in solvent I). Ratio of adenosine : pantothenic acid : sulfhydryl : phosphorus was 1:0.92:0.91:2.10; required, 1:1:1:2. Other properties are shown in Table III.

DISCUSSION

It has now been found that a larger amount of CoA is able to be synthesized together with other metabolites of pantothenic acid such as 3'-dephospho-CoA and 4'-phosphopantothenic acid in the reaction mixture containing pantothenic acid, cysteine, AMP (or ATP), and a kind of microorganism. This process may provide an effective method for the preparation of CoA and its biosynthetic intermediates. Especially, when labeled CoA is required, it probably provides more suitable method than the methods reported by several authors (52-55), because of its simplicity and its high conversion rate. Later, this method was applied to a synthesis of ^{14}C-CoA (56).

Several yeasts showed the activity of CoA accumulation upon an addition of AMP. Its phosphorylated product, ATP, which is necessary for the biosynthesis of CoA, might be formed through the process of glucose fermentation in the yeasts (34). While, the accumulation of CoA by bacteria was observed only when ATP was added.

Brevibacterium ammoniagenes IFO 12071 used here is well known as the organism which can synthesize not only nucleoside monophosphates, but also more complex nucleotides such as ATP, GTP, and so on (57,58). Moreover, Nakayama *et al.* (59) have reported a salvage synthesis of pyridine coenzymes from nicotinic acid or nicotinamide and adenine in the culture broth using *Brevibacterium ammoniagenes* ATCC 6872. Therefore, it seems to be possible to accumulate CoA directly from pantothenic acid, cysteine, and adenine or AMP in the culture broth using the similar system used in NAD synthesis. (This problem will be described in other sections).

Recently, Kuno *et al.* (29) have reported that several hydrocarbon assimilating microorganisms grown in pantothenate-supplemented medium accumulate intracellularly a relatively large amount of CoA. They also improved the method for the extraction and that for the purification of CoA. Alberts *et al.* (60) reported that the cellular concentration of CoA in pantothenate-requiring mutant of *Eschrichia coli* was directly related to the concentration of pantothenic acid in the medium. In the present study as indicated in Table II, several bacteria belonging to the genus of *Brevibacterium* contained higher amount of CoA. Therefore, it would be also possible to make the accumulation of CoA in higher level by culturing such bacteria in suitable pantothenate medium.

Section II.

Formation of Coenzyme A by Baker's Yeast[c)]

The possibility for the formation of CoA in a larger amount from pantothenic acid and cysteine was investigated with baker's yeast under the condition coupled with ATP-generating system. Effect of various factors affecting the accumulation of CoA was investigated. Among them, glucose concentration and inorganic phosphorus concentration were the most important factors for its accumulation. CoA was not accumulated without phosphorylation of AMP to ATP. Several cationic surfactants stimulated the accumulation of CoA. The amount of CoA accumulated reached about 200 μg per ml of the reaction mixture under the suitable reaction conditions.

INTRODUCTION

Microorganisms, especially yeasts, are known to be one of the most suitable sources of CoA because of their easy supply in a larger amount at a lower cost. The extraction and the purification of CoA from microbial cells have been reported by several workers (20,21,25,29). However, little has been reported on the accumulation of CoA with or without the addition of precursors such as pantothenic acid (10).

ATP is an essential cofactor for the biosynthesis of CoA from pantothenic acid (14). The phosphorylation of AMP has been investigated to obtain the higher yield of ATP with yeast cells by Tochikura *et al.* (34). In the preceding section, it has been demonstrated that several yeasts having the ability to phosphorylate AMP to ATP can accumulate CoA in the presence of pantothenic acid, cysteine, and AMP. Resting on the basis of this finding, the present investigation is undertaken with baker's yeast to estimate the conditions of the accumulation of CoA from pantothenic acid and cysteine for the coupling with ATP-generating system.

MATERIALS AND METHODS

Materials. Baker's yeast which was obtained from Oriental Yeast Co., Ltd., Tokyo, was used as an enzyme source throughout this work. Air-dried cells were prepared as described in Section I. Acetone-dried cells and ground cells were prepared according to the method reported by Tochikura *et al.* (34). An enzyme, which catalyzes the acetylation of sulfanilamide and is used for the determination of CoA, was prepared from pigeon liver according to the method of Novelli (61). Surfactants were kindly gifted from Sanyo Kasei Co., Ltd., Kyoto, and from Kao Soap Co., Ltd., Tokyo. Other chemicals used were the same as those in Section I.

Standard reaction mixture for CoA formation. The standard reaction mixture containing 167 μmoles of glucose, 15 μmoles of AMP, 10 μmoles of sodium pantothenate, 10 μmoles of cysteine, 200 μmoles of potassium phosphate buffer, pH 7.0, and 100 mg of dried cells with or without an addition of Cation-S (cetyldimethylbenzylammonium chloride) surfactant in a total volume of 1 ml. The reaction was carried out as described in Section I, unless otherwise stated.

Analytical methods. CoA was assayed by the phosphotransacetylase method of Stadtman *et al.* (43) using the enzyme of *Eschrichia coli* Crookes AKU 0001 as described in Section I, or by the sulfanilamide acetylation method of Kaplan and Lipmann (62). Glucose, fructose 1,6-diphosphate, and inorganic phosphorus were determined by the method of Somogyi (63), Roe (64), and Takahashi (65), respectively. Other saasys were

the same as those described in Section I.

RESULTS AND DISCUSSION

Effect of the preparation method of cells on the formation of CoA

As shown in Table I, the amount of CoA in the reaction mixture greatly increased only in the complete system. The omissions of each substrate gave a slight or no increased formation of CoA. It was found that the amount of CoA formed differed with yeast preparations which were treated by several methods (Table II). Ground cells, acetone-dried cells, and air-dried cells could form relatively large amounts of CoA. In the reaction system with intact cells, the amount of CoA did not increase after the incubation. It was also found that the addition of a certain surfactant to both systems of intact cells and acetone-dried cells was effective on the accumulation of CoA. These results may suggest that the formation of CoA occurred only when the injured cells were used. However, it is not clear, at present, whether CoA was formed in an extracellular system or not. Tochikura *et al.* (66) have obsreved that some enzymes concerning UDP-hexose formation from UMP and glucose under the fermentation conditions of air-dried cells of *Torulopsis candida* leak out into the supernatant of the reaction mixture. The similarity of the fermentation conditions in the present case to that of UDP-hexose formation suggests that the formation of CoA occurred extracellularly. In the following experiments, acetone-dried cells were used mainly because of its simplicity for the obtain of the constantly active preparations.

TABLE I. REQUIREMENT OF PANTOTHENIC ACID, CYSTEINE, GLUCOSE, AND AMP FOR CoA FORMATION

The complete reaction condition is described in the text. The reaction was carried out for 5.5 hr under the standard conditions except that each reaction component was omitted as indicated.

Condition	CoA found (μg/ml) before reaction	CoA found (μg/ml) after reaction
Complete	29	134
Omit pantothenic acid and cysteine	28	25
Omit pantothenic acid	30	33
Omit cysteine	35	66
Omit glucose	32	43
Omit AMP	33	60
Omit cells	0	0
Cells in potassium phosphate buffer, pH 7.0	28	20

TABLE II. EFFECT OF PREPARATION METHOD OF CELLS ON CoA FORMATION

The reactions were carried out for 6 hr under the standard conditions except that cells as enzyme source used were as indicated. The surfactant concentration was 1 mg per ml.

Cells used (100 mg/ml)	CoA formed (μg/ml)
Acetone dried cells	110
Acetone dried cells with Tween 60	105
Acetone dried cells with CPC	211
Intact cells	60
Intact cells with Tween 60	62
Intact cells with CPC	101
Air dried cells	88
Ground cells	102

CPC: cetylpyridinium chloride.

Effect of substrates on the formation of CoA

Glucose concentration and other sugars. The optimum concentration of glucose for the formation of CoA was 167 to 333 μmoles per ml of the reaction mixture (Fig. 1). At higher levels of glucose, the AMP added was mainly metabolized to yield adenosine and IMP accompanied with the repressed formation of CoA, although the fermentation of glucose was very active. The addition of sucrose or fructose as an energy source showed the same effect as glucose on the formation of CoA. These sugars probably entered to glycolytic pathway and resulted the formation of ATP. A weak fermentation and neither formation of ATP nor CoA were observed by an addition of other sugars such as galactose, xylose, glycerol, and α-glycerophosphate in place of glucose.

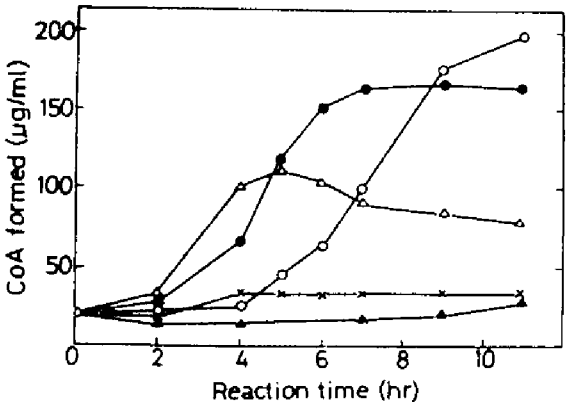

FIG. 1. Time Course for CoA Formation under Various Glucose Concentrations.

The reaction was carried out under the standard conditions except that glucose concentration was varied as follows: ×, without glucose; △, 83 μmoles/ml; ●, 167 μmoles/ml; ○, 333 μmoles/ml; ▲, 1111 μmoles/ml. Cation-S concentration was 0.5 mg/ml.

Pantothenic acid. The amount of CoA in the reaction mixture increased with an increased addition of pantothenic acid, but the rate of conversion from pantothenic acid to CoA decreased logarithmically. As shown in Table III, pantethine was about 2.0-fold as active as pantothenic acid. It seemed that the phosphorylation of pantethine (pantetheine) might occur and the resulted product entered to the pathway for the biosynthesis of CoA.

TABLE III. COMPARISON OF CoA FORMATION FROM PANTOTHENIC ACID AND PANTETHINE

The reaction was carried out for 7 hr under the standard conditions by an addition of each compound as indicated instead of pantothenic acid. Cation-S was also added to the reaction mixture at the concentration of 0.5 mg per ml.

Compound (μmoles/ml)	CoA formed (μg/ml)
Pantothenic acid (10)	115
Pantothenic acid (10) + pantethine (5)	270
Pantethine (1.5)	227
Pantethine (5)	225

Cysteine. The optimum concentration of cysteine for the formation of CoA was about 10 to 20 μmoles per ml of the reaction mixture. At higher levels of cysteine, the consumption of glucose and the formation of ATP were strongly inhibited. The decarboxylated product of cysteine, 2-mercaptoethylamine, which is also able to couple with 4'-phosphopantothenic acid to yield 4'-phosphopantetheine (14), was tested as a substrate for the CoA synthesis. It was about two-third as active as cysteine. Brown (14) reported that both of bacterial and mammalian coupling enzymes, which catalyze the coupling of 4'-phosphopantothenic acid with cysteine, can transform 4'-phosphopantothenic acid and 2-mercaptoethylamine to 4'-phosphopantetheine.

AMP concentration and other nucleotides. The amounts of CoA and ATP in the reaction mixture increased with an increase of AMP concentration. The amount of CoA reached more than 200 μg per ml of the reaction mixture with an addition of 54 μmoles per ml. When the direct precursor of CoA, ATP, was added instead of AMP at the start of the reaction, the formation of CoA was greatly stimulated. When glucose as an energy source was omitted, the ATP added was immediately degraded to IMP and the formation of CoA did not occur. Subsequently, the effects of other nucleotides and their related compounds on the formation of CoA were examined.

TABLE IV. FORMATION OF CoA USING SEVERAL NUCLEOTIDE-RELATED COMPOUNDS

The reaction was carried out for 7 hr under the standard conditions by an addition of each compound as indicated instead of AMP. Cation-S was also added to the reaction mixture at the concentration of 0.5 mg per ml.

Nucleotide-related compound added (10 μmoles/ml)	CoA formed (μg/ml)
Adenine	45
Adenosine	99
AMP	126
ADP	163
ATP	175
GMP	94
CMP	36
UMP	35
IMP	42

The result is summarized in Table IV. Of the adenine-containing compounds tested, ATP was the most effective, and ADP, AMP, and adenosine, which were well phosphorylated to ATP, were less effective than ATP in this oder. Neither the phosphorylation to ATP, nor the formation of CoA was observed with an addition of adenine instead of AMP. Among other nucleotides tested, only GMP had a little effect on the formation of CoA. In this case, GMP was well phosphorylated to GTP without the formation of ATP in a detectable amount. It might be possible that GTP derived from GMP functioned as a direct energy source of CoA synthesis in a similar manner to the ATP function. Brown (14) reported that CTP is an energy source for the coupling reaction between 4'-phosphopantothenic acid and cysteine in bacterial system. Kitajima *et al.* (67) reported that CMP can be phosphorylated to CTP under the similar fermentation condition of yeasts to that of the phosphorylation of AMP. In order to examine the supplementary effect of CTP, both AMP and CMP were added to the reaction mixture. The AMP and the CMP were well phosphorylated to the corresponding triphosphates, but further increased formation of CoA than that of AMP-only was not observed. This suggests that CTP may be specific only for bacterial enzyme, and the coupling enzyme of yeast may be resemble to mammalian enzyme rather than bacterial enzyme.

Other factors affecting the accumulation of CoA

The accumulation of CoA was accelerated with an increasing concentration of inorganic phosphorus up to 200 μmoles per ml of the reaction mixture. The amount of CoA formed in the reaction mixture reached 150 μg per ml at the concentration of 200 μmoles per ml. At higher concentrations of inorganic phosphorus, the consumption of glucose was strongly inhibited. While, at the lower concentrations, deamination of AMP was very accelerated. The feeding of inorganic phosphorus during the incubation did not show the influence to the accumulation of CoA.

The pH optimum of the reaction was between 6.5 and 7.0. The complete inhibition of glucose fermentation was observed at the higher pHs. The suitable temperature of the reaction for the accumulation of CoA was about 37°C. The accumulation of CoA was further accelerated by an aerobical condition accompanied with the activation of glucose fermentation and that of AMP phosphorylation.

Effect of divalent cations on the accumulation of CoA was investigated. Among the cations tested, Mg^{2+} and Mn^{2+} showed the stimulative effect on the accumulation of CoA, and shortened the reaction period. But Ca^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{2+} were all inhibitory. Magnesium ion probably acted as a cofactor not only in the process of the biosynthesis of CoA, but also in glycolytic pathway.

The results obtained above suggests that the formation of ATP, which probably occurred in preference to the accumulation of CoA, was affected by the factors such as glucose concentration, magnesium ion, and so on. Then, the accumulation of CoA might be brought about secondarily through the accumulation of ATP.

Effect of surfactant on the accumulation of CoA

A certain surfactant stimulated the accumulation of CoA as described above. Then, numerous surfactants which included cationics (12), anionics (8), nonionics (14), and amphoterics (4) were tested using the intact cells and the acetone-dried cells. It was found that all the cationics tested were effective in both yeast preparations. Among them, cetyltrimethylammonium chloride, cetylethyldimethylammonium bromide, cetylpyridinium chloride, and Cation-S gave the higher yields of CoA (Table V). The optimum concentration of the surfactant was observed at 1.0 to 1.2 mg per ml of the reaction mixture. At higher concentrations of the surfactant, both ATP and CoA were not formed because of its strong inhibitory effect on the fermentation of glucose. As shown in

TABLE V. EFFECT OF SURFACTANT ON CoA ACCUMULATION

The reaction was carried out under the standard conditions using acetone dried cells by an addition of each surfactant as indicated.

Surfactant (1 mg/ml)	CoA found (μg/ml) after			
	0	4	5.5	7 (hr)
None	25	68	95	40
Tween 60	24	74	90	55
Tween 80	30	-*	110	64
Span 60	25	80	45	37
Sodium oleate	25	131	80	55
Sodium stearate	26	55	87	59
KLS	24	31	36	31
SLP	25	82	60	38
CPC	25	41	-	190
LPC	28	-	55	105
STAC	21	55	135	145
CTAC	24	-	67	202
CTAB	20	133	-	153
LTAC	34	-	90	155
CEMAB	35	-	120	197
Cation-S	23	52	-	206
Lebon GM	24	45	111	191
Lebon 15	20	110	-	115

* not tested.

Abbreviations used: KLS, potassium laurylsulfate; SLP, sodium laurylphosphate; CPC, cetylpyridinium chloride; LPC, laurylpyridinium chloride; STAC, stearyltrimethylammonium chloride; CTAC, cetyltrimethylammonium chloride; CTAB, cetyltrimethylammonium bromide; LTAC, lauryltrimethylammonium chloride; CEMAB, cetylethyldimethylammonium bromide; Lebon GM, lauryldimethylbenzylammonium chloride; Lebon 15, sodium alkyldi(aminoethyl)-glycine.

Fig. 2, the additions of the surfactant at early reaction periods was more effective than those at the later periods. The stability of pure CoA solution was not affected by the presence of the surfactant. It has been reported that some surfactants bring the serious affect to enzyme structures or enzyme actions (68-70). It has been also observed that several surfactants give effective actions to the extraction of compounds from microbial cells (71), and that some enzymes or some metabolic products leak out in the presence of surfactant (72-74). In the present case, however, it is difficult to decide which the effect was caused (repression of enzymatic degradation of CoA? stimulation of CoA synthesizing enzymes? or simple extractive effect to the reaction products?).

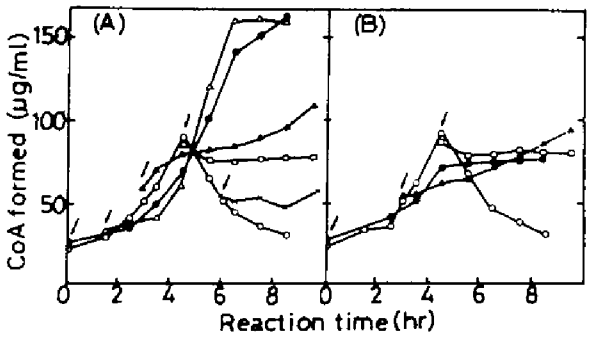

FIG. 2. Effect of Cation-S Surfactant on CoA Accumulation.

The reaction was carried out under the standard conditions except that Cation-S was added at the time indicated by the arrows at the concentration of 1.5 mg/ml (A) and 2.5 mg/ml (B), respectively. O : control run (without Cation-S).

Time course of the reaction

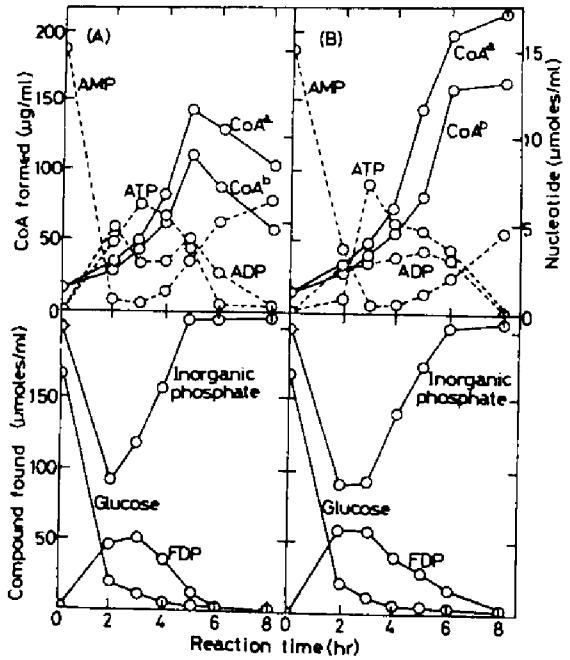

FIG. 3. Formation and Consumption of Several Compounds in the Reaction Mixture.

The reaction was carried out under the standard conditions with (A) or without (B) 1.5 mg of Cation-S surfactant. CoA was assayed by the sulfanilamide acetylation method of Kaplan and Lipmann (62) (a); and by the phosphotransacetylase method of Stadtman *et al.* (43) (b). FDP: fructose 1,6-diphosphate.

The time course of the formation of products and the consumption of substrates in the reaction mixture is shown in Fig. 3. The consumption of glucose, inorganic phosphorus, and AMP occurred first, then the accumualtion of fructose 1,6-diphosphate and ATP, and finally CoA accumulation. The remarkable decrease of inorganic phosphorus in the reaction mixture prior to the accumulation of CoA suggests that it might be incorporated into fructose 1,6-diphosphate, a key intermediate in the glycolytic pathway, which might be, then, further metabolized to yield further ATP.

When the amount of CoA formed in the reaction mixture was assayed by both of the phosphotransacetylase method of Stadtman *et al.* (43) and the sulfanilamide acetylation method of Kaplan and Lipmann (62), the amount of CoA detected was higher in the latter case than that in the former (Fig. 3). It seems that the result might be attributed to the lower specificity of the latter method for the intermediates of CoA biosynthesis.

Section III.

Formation of Coenzyme A by *Brevibacterium ammoniagenes* IFO 12071[d)]

Several conditions for the formation of CoA from pantothenic acid and cysteine in the presence of ATP were investigated using *Brevibacterium ammoniagenes* IFO 12071. The higher activity of the formation of CoA was observed with the cells grown in acetate media. The effects of the substrate concentration, phosphate buffer concentration, pH, and cell concentration on the formation of CoA were examined and the amount of CoA formed reached a maximum value of 1.2 mg per ml of the reaction mixture. Several surfactants belonging to cationics and anionics brought about 1.8-fold stimulation of CoA formation. The considerable accumulation of CoA in the culture broth during the growth was also observed.

INTRODUCTION

In the previous sections, it has been demonstrated that dried cells of several kinds of microorganisms, especially, *Brevibacterium ammoniagenes* IFO 12071, formed a larger amount of CoA and its biosynthetic intermediates from pantothenic acid and cysteine in the presence of AMP or ATP. The isolation and the characterization of CoA, 3'-dephospho-CoA, and 4'-phosphopantothenic acid formed in the reaction mixture were also described.

In the present section, the effects of various factors on the formation of CoA by *Brevibacterium ammoniagenes* IFO 12071 are investigated.

MATERIALS AND METHODS

Microorganism and cultivation. *Brevibacterium ammoniagenes* IFO 12071 was used throughout this work. The culture techniques were the same as described in Section I, unless otherwise stated.

Preparation of dried cells. Dried cells of the bacterium were prepared as described in Section I.

Chemicals. 4'-phosphopantothenic acid and 3'-dephospho-CoA were prepared as described in Section I. Other chemicals used were the same as those described in Section I.

Standard reaction condition for CoA formation. The standard reaction mixture contained 10 μmoles of sodium pantothenate, 10 μmoles of cysteine, 15 μmoles of ATP, 10 μmoles of $MgSO_4$, 150 μmoles of potassium phosphate buffer, pH 6.0 or 7.0, and 100 mg of air-dried cells of *Brevibacterium ammoniagenes* in a total volume of 1 ml. All other conditions were the same as those described in Section I, unless otherwise stated.

Analytical methods. The analytical methods employed here were the same as those described in Section I.

RESULTS

Formation of CoA by air-dried cells

Culture conditions for the preparation of cells. In order to know whether the activity of CoA synthesis is varied by growth conditions or not, the cultivations were carried out with various media. The stronger activity of CoA formation was found in the cells grown in glucose media and in acetate media. Results are summarized in Table I. The cells from acetate-NH_4Cl medium consumed about 40% of the pantothenic acid added and accumulated 1.2 mg of CoA per ml of the reaction mixture (The conversion rate to the pantothenic acid added was 15.7%.) under the standard conditions. However, the growth of the organism in acetate media was relatively slow and weak, then the cells grown in glucose-peptone medium were mainly used in the

TABLE I. EFFECT OF CULTURE CONDITIONS FOR THE PREPARATION OF CELLS ON CoA FORMATION

The cultivation was carried out aerobically at 28°C for 60 hr with the medium which composed of 3 g of carbon source as indicated, 0.5 g of nitrogen source as indicated, 0.2 g of K_2HPO_4, 0.1 g of KH_2PO_4, 0.2 g of NaCl, 0.02 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of yeast extract, and 100 ml of tap water, pH 7.0. *n*-Paraffin used contained C_{13}, 0.4%; C_{14}, 68.1%; C_{15}, 29.9%; C_{16}, 0.8%; and aromatics, 1.3%.

The activity of CoA formation was measured as described in the text with the reaction time for 5 hr.

Medium[a)]	CoA found (μg/ml) before reaction	CoA found (μg/ml) after reaction
Glucose-peptone	15	566
Glucose-urea	15	500
Glucose-NH_4Cl	10	573
Sodium acetate-peptone	16	790
Sodium acetate-NH_4Cl	55	860
Glycerol-peptone	15	265
n-Paraffin-peptone	5	0

a) A very weak growth or no growth was obserbed in sodium acetate-urea, glycerol-urea, glycerol-NH_4Cl, *n*-paraffin-urea, and *n*-paraffin-NH_4Cl under the conditions tested.

following experiments. The relationship between the activity of CoA synthesis and the growth phase of the organism in glucose-peptone medium was investigated. For this experiment, cells were cultivated at 28°C for 15 to 108 hr. The activity of CoA synthesis was almost constant in the range tested.

Reaction conditions. Several reaction conditions for CoA formation were investigated with the cells grown in glucose-peptone medium for 24 hr.

i) Effects of pantothenic acid, cysteine, and ATP concentration. As shown in Fig. 1, at the concentration of 1 μmole of pantothenic acid per ml of the reaction mixture about 85% of the pantothenic acid added was converted to CoA. The amount of CoA increased with an increased addition of pantothenic acid up to 10 μmoles per ml. Further addition of pantothenic acid brought a decrease of CoA formation. On the other hand, cysteine requirement for the maximum formation of CoA was more than 10 μmoles per ml. The amount of CoA formed in

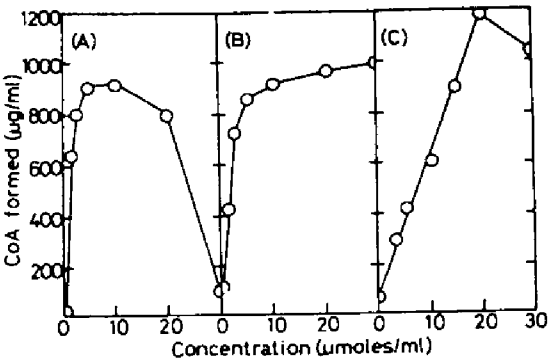

FIG. 1. Effect of Substrate Concentration on CoA Formation.

Each reaction was carried out under the standard conditions for 5 hr except that pantothenic acid (A), cysteine (B), or ATP (C) was varied at the concentration as indicated.

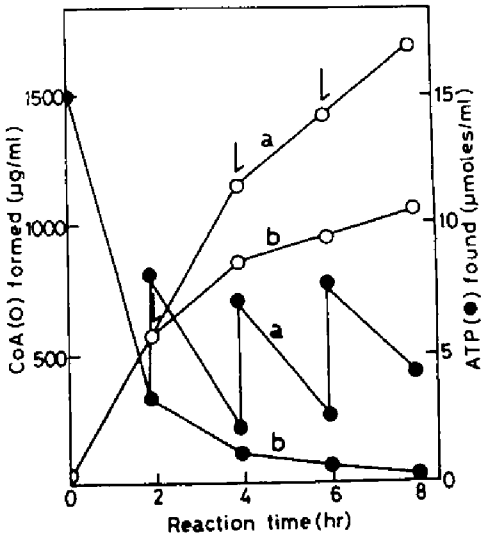

FIG. 2. Effect of ATP Supplying on CoA Formation.

The reaction was carried out under the standard conditions except that 5 μmoles of ATP were added at the time indicated by the arrows in the case of (a). (b) is a control run.

the reaction mixture increased linearly up to 20 μmoles of ATP per ml and reached a maximum value of 1.2 mg per ml. It seemed, consequently, ATP concentration limited the formation of CoA rather than those of pantothenic acid and cysteine under the conditions tested. The supply of ATP during the incubation brought the increase in the amount of CoA formed (Fig. 2). Pierpoint *et al.* (75) reported that the phosphorylation of

pantothenic acid by dried cells of *Lactobacillus arabinosus* was 1.4-fold activated by an addition of NaF, and they suggested the presence of ATPases in the cells. In the present case, however, the reaction was not affected by an addition of NaF or KCN as phosphatase inhibitor.

ii) Phosphate buffer concentration. Relatively higher activity was found beween 90 and 200 mM of potassium phosphate buffer, pH 6.0. At lower phosphate concentrations, the conversion of the ATP added to adenine and adenosine was observed, while these compounds were not detected from the reaction mixture at the concentrations of 90 mM and over. At the higher concentrations of phosphate, the ATP added remained without its utilization for CoA formation.

iii) pH. It was observed that the reaction had a pH optimum between 6 and 6.5. Of the buffers used, potassium phosphate buffer was the most suitable for the formation of CoA, while Tris buffers were rather inhibitory.

iv) Cell concentration. The cell concentrations of 100 to 150 mg per ml of the reaction mixture were effective on the formation of CoA. It seemed that degradation of the product was caused at the concentration of 200 mg per ml and by longer reaction time. The lower concentrations of the cells (less than 40 mg per ml) gave only negligible formation of CoA. At least, 50 mg of the cells per ml were required for the formation of CoA.

v) Metal ion requirement. Among the cations tested, Mg^{2+} showed a slight acceleration for the formation of CoA. Magnesium ion is known to be a cofactor throughout the pathway for the biosynthesis of CoA. The result obtained here may suggest that the sufficient amount of Mg^{2+} to synthesize CoA was present in the cells. It was also observed that the formation of CoA was strongly inhibited by the additions of 10 μmoles of Cu^{2+}, Co^{2+}, and EDTA per ml, respectively.

vi) Temperature. The optimum temperature of the reaction was in the range from 37 to 42°C. Under higher temperatures than 45°C, the formation of CoA was completely repressed.

vii) Effect of various compounds relating to CoA biosynthesis. Among the nucleotides tested, only ATP was active for the formation of CoA. CTP has been known as an energy source for the coupling of 4'-phosphopantothenic acid with cysteine in bacterial systems such as *Eschrichia coli* and *Proteus morganii* (14). In the present case, the supplementary effect of CTP was very slight when compared with that of ATP-only in this experiment (Table II). While, 4'-phosphopantothenic acid was completely converted to 4'-phosphopantetheine by the cell suspension of *Brevibacterium ammoniagenes* IFO 12071 in the presence of CTP and cysteine, but not ATP and cysteine, which will be described in other sections. Among the sulfhydryl donor tested, L-cystine was active as well as L-cysteine, but D-cysteine was not active. 2-Mercaptoethylamine was inactive under the conditions tested. As shown in Table III, pantethine, 4'-phosphopantothenic acid, and 3'-dephospho-CoA were also converted to CoA with higher conversion rates than that of pantothenic acid. Pantothenyl alcohol, which is an alcohol analog corresponding to pantothenic acid, was not converted to CoA.

TABLE II. EFFECT OF NUCLEOTIDES ON CoA FORMATION

The reaction was carried out for 6 hr under the standard conditions by an addition of each nucleotide instead of ATP.

Nucleotide (μmoles/ml)	CoA formed (μg/ml)
None	221
ATP (15)	815
ADP (15)	281
AMP (15)	215
GTP (15)	362
GDP (15)	314
CTP (15)	367
UTP (15)	358
ITP (15)	367
ATP (15) + CTP (10)	928
ATP (15) + CTP (5)	896

TABLE III. FORMATION OF CoA FROM PANTOTHENATE DERIVATIVES

The reaction was carried out for 8 hr under the standard conditions by additions of 2 mg of sodium laurylbenzenesulfonate and of each substrate instead of pantothenic acid.

Compound (μmoles/ml)	CoA formed (μg/ml)
Pantothenic acid (10)	2051
Pantothenyl alcohol (10)	79
Pantethine (5)	2480
4'-Phosphopantothenic acid (10)	3978
3'-Dephospho-CoA (10)	6997

Formation of CoA by intact cell system and growing cell system

Effect of surfactant on several cell preparations. The stimulative effect of surfactants on CoA accumulation by baker's yeast was described in Section II. In this experiment, several surfactants were tested. The result is shown in Table IV. The remarkable stimulation to the accumulation of CoA by some of them belonging to cationics and anionics was observed. On the other hand, nonionics employed here showed no simulative effect. The concentrations of 1 to 2 mg of sodium laurylbenzenesulfonate or sodium laurylsulfate per ml gave the good CoA accumulation. At the concentration of 2 mg per ml, the amount of CoA formed rose to about 1.3-fold when compared with no addition of surfactant. Further addition of surfactant caused the inhibitory effect. This stimulative effect of surfactant was also observed in intact cell system, acetone-dried cell system, and this effect was most remarkable in intact cell system. In intact cell system, it was found that the fomation of CoA and

TABLE IV. EFFECT OF SEVERAL SURFACTANTS ON CoA FORMATION

The reaction was carried out under the standard conditions for 8 hr with an addition of each surfactant as indicated.

Surfactant (1 mg/ml)	CoA formed (μg/ml)
None	1025
Span 60	1031
Tween 80	1011
Cetylbenzyldimethylammonium chloride	1028
Stearyltrimethylammonium chloride	1086
Cetylpyridinium chloride	1690
Sodium laurylbenzenesulfonate	1875
Sodium laurylsulfate	1725
Sodium cetylsulfate	1390
Sodium laurylphosphate	1294
Sodium laurate	1269

TABLE V. EFFECT OF SURFACTANT ON SEVERAL CELL PREPARATIONS

The reaction mixture containing 5 μmoles of sodium pantothenate, 10 μmoles of cysteine, 15 μmoles of ATP, 10 μmoles of $MgSO_4$, 150 μmoles of potassium phosphate buffer, pH 6.0, and each cell preparation as indicated was incubated with or without 1 mg of sodium laurylsulfate (SLS) for 6 hr at 37°C in a total volume of 1 ml. The amounts of the cells added into the reaction mixture were 54 mg, 100 mg, and 100 mg in A, B, and C, respectively. In the case of D, the reaction mixture containing 0.1 μmole of sodium pantothenate, 5 μmoles of ATP, 2 μmoles of cysteine, 1 μmole of $MgSO_4$, 30 μmoles of potassium phosphate buffer, pH 6.0, and 0.3 ml of cell free extract was incubated with or without SLS for 2 hr at 37°C in a total volume of 1 ml.

Preparation	SLS added (mg)	CoA formed (μmoles)	Remaining pantothenic acid (μmoles)	Remaining ATP (μmoles)
A. Intact cells	0	0.14	4.80	14.0
"	1.0	1.18	0.60	0.5
B. Air dried cells	0	1.19	1.30	0.5
"	1.0	2.05	0.20	0.5
C. Acetone dried cells	0	1.60	0.95	0.3
"	1.0	1.75	0.54	0.0
D. Cell free extract	0	0.10	0.00	0.0
"	0.2	0.09	0.00	0.0
"	1.0	0.03	0.05	0.0

the consumption of ATP did not occur without the addition of surfactant. On the other hand, the addition of surfactant brought rather inhibitory effect to the formation of CoA in cell free system (Table V). These facts suggest that CoA might be accumulated only by the injured cell preparations. In the experiment to examine the extracellular excretion of the enzymes relating to CoA biosynthesis, the intact cells were contacted with sodium laurylsulfate in 0.05 M potassium phosphate buffer, pH 6.0 for 2 hr at 37°C, and then the activity of pantothenate kinase in the supernatant was measured. But the significant activity was not detected.

Direct accumulation of CoA in culture broth. Because the formation of CoA occurred using the intact cells and surfactant system as demonstrated above, an attempt to accumulate CoA in the culture broth during the growth with an addition of adenine derivative was briefly done. The bacterium produced a large amount of CoA when it was grown in the medium containing CoA precursors in the presence of cetylpyridinium chloride surfactant as shown in Table VI. The accumulation of CoA with the growing cells in the culture broth will be investigated in detail in the following section.

TABLE VI. ACCUMULATION OF CoA IN CULTURE BROTH DURING THE GROWTH

Cultivation was carried out in 500 ml shaking flask containing 20 ml of the medium of Tanaka *et al.* (58), which contained 2 g of glucose, 0.12 g of urea, 0.2 g of KH_2PO_4, 0.2 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of yeast extract, 2 mg of $CaCl_2 \cdot 2H_2O$, and 0.6 μg of biotin. After 3 day cultivation at 28°C, 30 mg of sodium pantothenate, 40 mg of cysteine, 40 mg of adenine derivative as indicated, and 20 mg of cetylpyridinium chloride (CPC) were added to the medium, except that in the case of A and D, the adenine derivative was added before the cultivation, and in the case of C, CPC was omitted. Then the cultivation was continued for a further 2 days. After boiling the culture for 10 min and removing the cells, CoA in the culture broth was assayed as described in the text.

Condition	CoA formed (mg/ml)	pH after 3 day culture
A. adenine + CPC	0.0	7.6
B. adenine + CPC	1.2	6.4
C. AMP	0.3	6.0
D. AMP + CPC	2.9	6.0
E. AMP + CPC	2.1	6.0
F. ATP + CPC	2.5	6.0

DISCUSSION

The amount of CoA formed by the air-dried cell system of *Brevibacterium ammoniagenes* reached a maximum value of 2.0 mg per ml of the reaction mixture upon an addition of suitable concentration of surfactant. Furthermore, it was observed that several mg of CoA per ml of the culture broth were accumulated during the growth in the presence of a surfactant.

As described in the preceding section, the formation of CoA with yeast cell system was stimulated by cationic surfactants, but not by anionic ones. While, the stimulation for CoA formation with bacterial cell system was observed not only by an addition of cationic surfactant but also by that of anionic one. The effects of surfactants on metabolism of microorganisms have been reported in several microbial processes such as the glutamic acid fermentation, nucleic acid-related compounds production, and so on (73,74,76,77). Nara *et al.* (73) reported the leakage of ribose 5-phosphate and the enzymes for salvage synthesis of IMP into the liquid medium in the presence of certain cationic surfactant. In the case presented here, it seemed that the stimulative effect of surfactants on the formation of CoA was caused by the changes of the permeability of the substrates and the products rather than the leakage of CoA synthesizing enzymes.

ATP is required as an essential energy source and a substrate for the CoA biosynthesis (14). As demonstrated above, ATP concentration was one of the most important factors for the CoA formation in the dried cell system. While, in growing culture system, the accumulation of CoA was observed by the addition of AMP. This suggests that an efficient accumulation of CoA in the culture broth was brought through the

regeneration of the ATP used.

The cells grown in acetate media showed the higher activity of CoA formation than those grown in glucose media. This may be caused by the differences between the anabolic pathways of acetic acid and glucose.

Section IV.

An Improved Method for the Fermentative Production of Coenzyme A from Pantothenic Acid, Cysteine, and 5′-AMP[e,f)]

The cultivation of *Brevibacterium ammoniagenes* IFO 12071 with pantothenic acid, cysteine, and AMP gave CoA in a high yield. The organism was stabilized by repeated single colony isolations. The culture conditions optimal for the production of CoA were investigated, and the yield of CoA in the culture broth reached more than 3 mg/ml. The advantages and disadvantages of the present method were discussed by comparing them with the original dried cell method.

INTRODUCTION

Several successful processes for the production of various 5′-ribonucleotides using microorganisms have been reported (78). Kinoshita and his coworkers (58, 59,79-81) have reported that ribotides of purines, pyrimidines, and their analogs were produced through a salvage pathway in *Brevibacterium ammoniagenes* ATCC 6872 and other bacteria. Ogata, Tochikura, and their coworkers (82-85) have reported the production of various nucleotide coenzymes under fermentation or respiration conditions of yeasts. These reports strongly suggest the possibility of a higher production of CoA with microorganisms. And in the previous sections, the author has demonstrated higher productions of CoA from pantothenic acid, cysteine, and ATP or AMP by dried cells of *Brevibacterium ammoniagenes* IFO 12071 and by dried cells of baker's yeast.

This section describes an application of a general and satisfactory process for nucleotide production by Kinoshita *et al.* to the production of CoA.

MATERIALS AND METHODS

Microorganism. *Brevibacterium ammoniagenes* IFO 12071 was used.

Chemicals. CoA (92% pure) used as a standard was prepared by incubating pantothenic acid, cysteine, and ATP with dried cells of *Brevibacterium ammoniagenes* IFO 12071 as described in Section I. Acetylphosphate was prepared by the method of Avison (42). Surfactants were provided by Sanyo Kasei Co., Ltd., Kyoto; Nikko Chemicals Co., Ltd., Tokyo; and Kao Soap Co., Ltd., Tokyo. Other chemicals were obtained from commercial sources.

Culture methods. Forty ml of a seed culture medium containing 0.4 g of glucose, 0.6 g of peptone, 0.12 g of K_2HPO_4, 0.08 g of NaCl, and 0.008 g of $MgSO_4 \cdot 7H_2O$, pH 7.0, were poured into a 500 ml shaking flask and the inoculated medium was shaken for 24 hr at 28°C. Two ml of the grown seed were inoculated into 20 ml of the fermentation medium in a 500 ml shaking flask. The fermentation medium, which was derived from the medium of Tanaka *et al.* (58), contained 2 g of glucose, 0.12 g of urea, 0.2 g of yeast extract, 0.4 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 0.04 g of AMP in 20 ml of deionized water. The initial pH of the medium was adjusted to 7.6. Urea was autoclaved separately. All fermentations were run at 28°C for 5 to 7 days on a reciprocal shaker (Satake Kagaku, 951), which was set for 140 oscillations per min with an amplitude of 14 cm unless otherwise stated. To the 3 day culture, 0.04 g of calcium pantothenate, 0.04 g of cysteine, and 0.02 g of cetylpyridinium chloride were added, and the cultivation was continued for a further 2 to 4 days. The cultured broth was boiled for 3 min and centrifuged. The supernatant was employed in the determination of the products.

Analytical methods. The analytical methods employed here were the same as

those described in Section I.

RESULTS

Selection of an active strain from Brevibacterium ammoniagenes IFO 12071

First, the original strain IFO 12071, which produced 1-2 mg/ml of CoA at low frequency, was stabilized by repeated single colony selections in order to obtain a constantly active strain. The original strain was inoculated on agar-plates containing the fermentation medium, and incubated at 28°C for 3 days. One hundred and twenty-five strains were derived from the original strain, and 46 strains produced more than 2 mg/ml of CoA. One of them was found to produce about 5 mg/ml of CoA (Table I).

TABLE I. SELECTION OF AN ACTIVE STRAIN FROM *BREVIBACTERIUM AMMONIAGENES* IFO 12071 BY SINGLE CELL ISOLATION

Fermentation was carried out as described in the text.

Productivity CoA mg/ml[a)]	Number of strain	%
5.0 - 3.5	2	2
3.4 - 3.0	8	6
2.9 - 2.5	17	14
2.4 - 2.0	19	15
1.9 - 1.5	14	11
1.4 - 1.0	7	6
0.9 - 0.5	3	2
0.4 - 0	55	44
Total	125	100

a) CoA assays were made for 7 day cultures.

Culture conditions

Glucose and other sugars. Figure 1 shows that the optimum level of glucose in the fermentation medium for the production of CoA was about 10-12%. At lower levels of glucose, production of ATP and CoA was scarcely observed at all, though the cell growth was rapid and strong. A weak growth and no production of CoA was observed when glucose was replaced by other sugars (sucrose, fructose, mannose, maltose, and xylose).

Nitrogen sources and nutrients. Several nitrogen compounds and nutrients

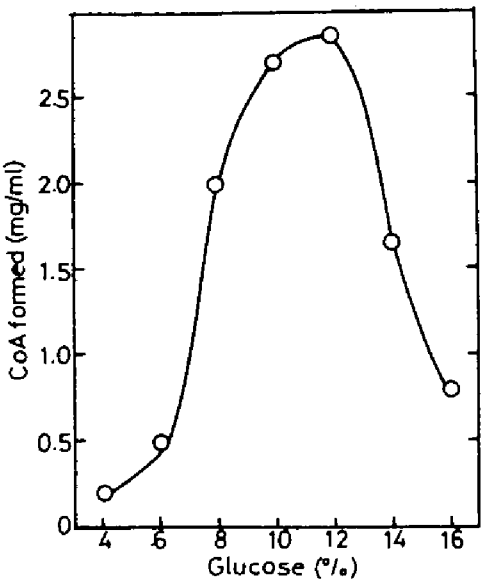

FIG. 1. Effect of Glucose Level on Production of CoA.

Fermentation was carried out as described in the text except for glucose concentration. CoA assays were made for 6 day cultures.

listed in Table II were tested for the production of CoA. Only combinations of urea and natural nutrients gave a good production of CoA. Either omission of urea or replacement of urea with other inorganic nitrogen compounds gave a decreased production of CoA, though the cell growth was rapid and strong. Combinations of urea and inorganic nitrogen compounds as listed in Table II were also ineffective for the production of CoA as well as for the cell growth. For good production, a 0.6% or higher level of yeast extract was required, when the effect of the yeast extract level on the production of CoA was examined.

Inorganic phosphate and magnesium sulfate. The levels of both inorganic phosphate and magnesium sulfate in the fermentation medium affected the production of CoA. The optimum level of inorganic phosphate was 1.5-2.0%, and that of magnesium sulfate was about 1.2%.

Pantothenic acid and cysteine. The amount of CoA produced increased with an increased addition of pantothenic

TABLE II. EFFECT OF NITROGEN SOURCES AND NUTRIENTS ON PRODUCTION OF CoA

Fermentation was carried out as described in the text except for nitrogen and nutrient.

Nitrogen source or nutrient (%)	CoA formed (mg/ml)a)	Growth b)
Yeast extract (1.0)	0.2	+++
Urea (1.6)	0.0	-
" (1.6) + yeast extract (0.1)	0.0	-
" (0.1) + " (1.6)	0.2	+++
" (0.6) + " (1.0)	2.4	+++
" (0.6) + meat extract (1.0)	3.3	+++
" (0.6) + corn steep liquor (1.0)	0.1	+++
" (0.6) + peptone (1.0)	3.6	+++
" (0.6) + mixed amino acid (1.0)	1.2	+++
" (0.6) + $(NH_4)_2HPO_4$ (1.0)	0.0	+
" (0.6) + $(NH_4)_2SO_4$ (1.0)	0.0	+
" (0.6) + NH_4NO_3 (1.0)	0.0	+

a) CoA assays were made for 5 day cultures.
b) Growth was checked for 3 day cultures: +++, strong; +, weak; -, no growth.

acid, and reached a maximum level (4.8 mg/ml) at 2-4 mg/ml of pantothenic acid. Further addition of pantothenic acid gave a decreased production of CoA (Fig. 2). The cysteine requirement for maximum production of CoA was 1-3 mg/ml (Fig. 3).

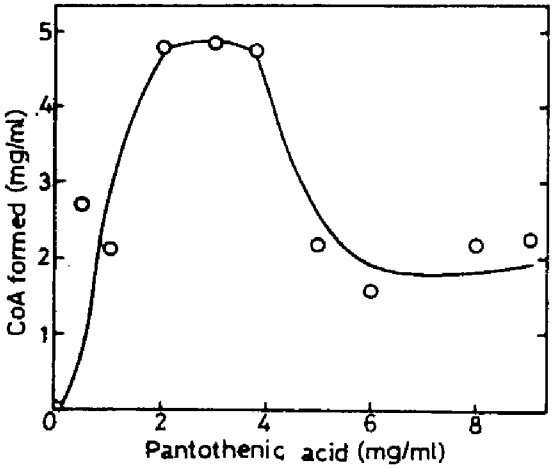

FIG. 2. Effect of Pantothenic Acid Level on Production of CoA.

Fermentation was carried out as described in the text except for calcium pantothenate concentration. CoA assays were made for 5 day cultures.

AMP and its related compounds. As to the AMP level, the production of CoA was observed over a wide concentration range of AMP (1-9 mg/ml), when yeast

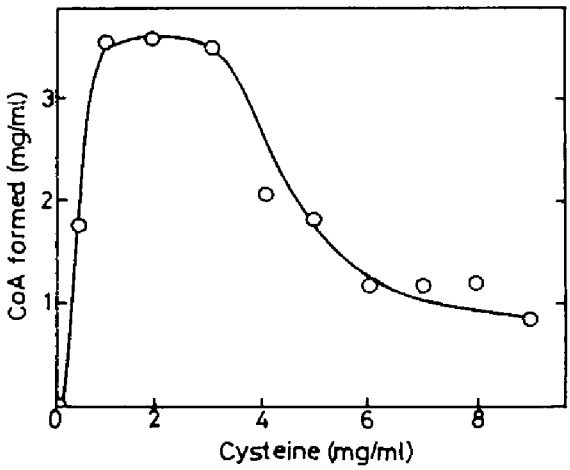

FIG. 3. Effect of Cysteine Level on Production of CoA.

Fermentation was carried out as described in the text except for cysteine concentration. CoA assyas were made for 5 day cultures.

extract was used as a nutrient. On the other hand, a higher production of CoA was observed over relatively limited ranges of AMP (2-3 mg/ml), when poptone was used as a nutrient, and a maximum production of CoA (5.5 mg/ml) occurred at 3 mg/ml of AMP. A mixed addition of peptone and yeast extract also gave a higher production of CoA (Fig. 4).

The production of CoA with adenine or adenosine replacing AMP was examined. Either adenine or adenosine repressed the cell growth and gave no production of CoA, when added before the culture.

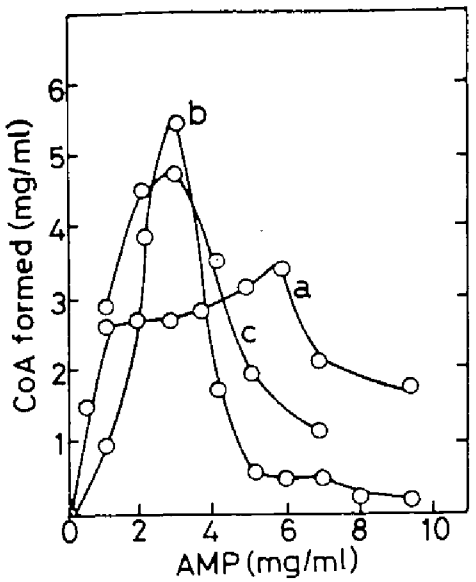

FIG. 4. Effect of AMP Level on Production of CoA.

Fermentation was carried out as described in the text except for AMP concentration and nutrient: a, yeast extract 1.0%; b, peptone 1.0%; c, peptone 1.0% + yeast extract 0.3%. CoA assays were made for 5 day cultures.

TABLE III. PRODUCTION OF CoA FROM ADENINE OR ADENOSINE

Fermentation was carried out as described in the text except that AMP was replaced with adenine or adenosine. AMP-supplemented medium was used for the control run.

Compound added (2 mg/ml)	Addition time	CoA formed (mg/ml)a)
Adenine	before culture	0.2
	after 3 days	1.5
Adenosine	before culture	0.1
	after 3 days	1.8
Control (AMP)	before culture	2.5

a) CoA assays were made for 7 day cultures.

However, additions made to 3 day cultures gave a good production of CoA (Table III).

Brown (14) reported that CTP is required for the coupling of 4'-phosphopantothenic acid and cysteine in a bacterial system. In order to examine the supplementary effect of CTP, both AMP and CMP (or CTP) were added to the culture. But further increased production of CoA was not observed, though consumption of pantothenic acid was somewhat rapid.

Surfactants. The stimulative effect of surfactants on the production of CoA by dried cells of baker's yeast and of *Brevibacterium ammoniagenes* IFO 12071 was described in the preceding sections. In this experiment, various surfactants were tested, and a remarkable stimulation of the production of CoA by certain surfactants belonging to the class of cationics and amphoterics was observed. One of them, Lebon 15, gave the best production of CoA (Table IV). The anionics and nonionics employed here were ineffective, though most anionics were very effective in the system using dried cells or washed cells of *Brevibacterium ammoniagenes* IFO 12071 (see Section III).

TABLE IV. EFFECT OF SURFACTANT ON PRODUCTION OF CoA

Fermentation was carried out as described in the text except for the surfactant.

Surfactant (1 mg/ml)	CoA formed (mg/ml)a)
No addition	0.0
Cetylpyridinium chloride	2.4
Laurylpyridinium chloride	2.9
Cetyltrimethylammonium bromide	1.4
Cetylethyldimethylammonium chloride	2.6
Stearyltrimethylammonium chloride	2.7
Cetylbenzyldimethylammonium chloride	2.9
Laurylbetaine	2.6
Lebon 15*	4.4
Sodium laurate	0.1
Potassium laurylsulfate	0.0
Sodium di-2-ethylhexylsulfosuccinate	0.2
Laurylsarcosinate	0.8
Tween 20	0.0
Tween 80	0.0
Span 20	0.0
Span 40	0.0

a) CoA assays were made for 5 day cultures.
* Sodium alkyldi(aminoethyl)glycine.

Aeration. The strain was cultured in a 500 ml shaking flask with varying volumes of the fermentation medium. The stable production of CoA was observed in flasks containing 10-60 ml of the medium (Fig. 5). When the fermentation was carried out at 120 oscillations per min and an amplitude of 7 cm, the

cell growth and production of CoA were greatly repressed (Table V).

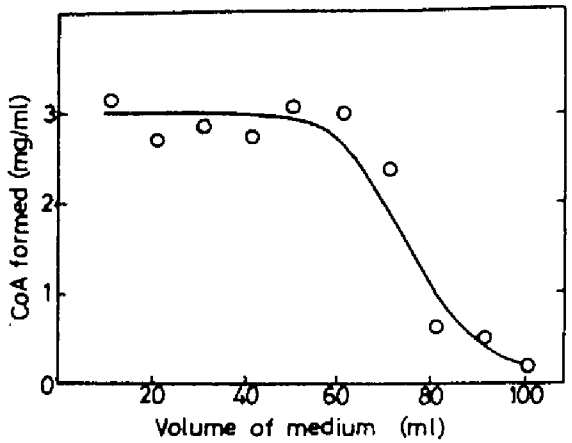

FIG. 5. Effect of Culture Volume on Production of CoA.

Fermentation was carried out as described in the text except for the volume of medium. Each seed volume was 10%. CoA assays were made for 7 day cultures.

TABLE V. EFFECT OF SHAKING ON PRODUCTION OF CoA

Fermentation was carried out as described in the text except for conditions indicated.

Shaking condition	Volume of medium (ml)	CoA formed (mg/ml)[a]	Growth (mg/ml)[b]
Oscillation 140/min Amplitude 14 cm	20	2.7	25.0
	40	2.5	24.5
Oscillation 140/min Amplitude 7 cm	20	0.2	15.5
Oscillation 120/min Amplitude 7 cm	20	0.0	8.5
Oscillation 100/min Amplitude 7 cm	20	0.0	7.7

a) CoA assays were made for 6 day cultures.
b) Growth was checked for 3 day cultures.

Time course of CoA production

An example of the time course of CoA production under optimum conditions is given in Fig. 6. First, phosphorylation of AMP to ATP, then consumption of pantothenic acid occurred, and finally, CoA was gradually produced. The maximum accumulation of CoA was attained on the 6th day.

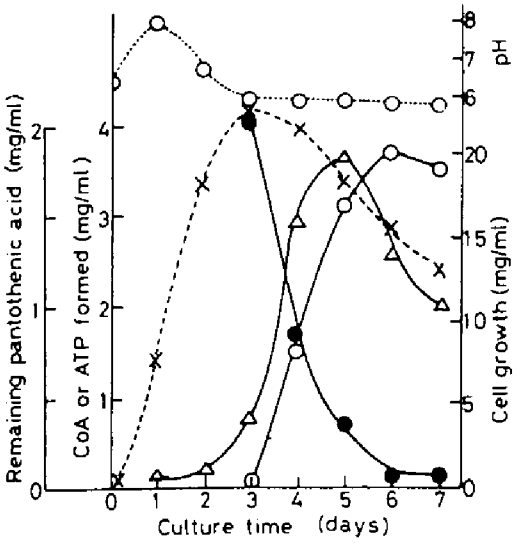

FIG. 6. Time Course for CoA Production.

Fermentation was carried out as described in the text except that the nutrients used were peptone (0.8%), yeast extract (0.5%), and biotin (30 µg/liter). O—O, CoA; ●—●, pantothenic acid; △—△, ATP; ×-×, cell growth; O··O, pH.

DISCUSSION

General and practical methods for the synthesis of nucleotide derivatives from the corresponding base, nucleoside, or nucleotide, using microorganisms, fall roughly into two groups: (I) Incubation of dried cells of various microorganisms, especially yeasts, with a high concentration of glucose and inorganic phosphorus gives nucleoside di- and triphosphates or sugar nucleotides such as ATP (34), UTP and UDP-glucose (82), GTP and GDP-mannose (83), and so on, from the corresponding nucleoside monophosphate in good yields. Examples of the variations of this method are the synthesis of CoA as described in the previous sections and cytidine coenzymes (84,85). (II) Cultivation of microorganisms, especially bacteria, in a medium containing high concentrations of glucose and salts also produces nucleoside mono-, di-, and triphosphates from the corresponding bases in the culture broth. Examples of this method are the synthesis of IMP (79), ATP (58),

NAD (59), and so on.

As to the synthesis of CoA, incubation of pantothenic acid, cysteine, and AMP or ATP with dried cells of baker's yeast or *Brevibacterium ammoniagenes* IFO 12071 gave an excellent yield of CoA as described in the previous sections. This method is simple, rapid, and clean, though it requires the dried cell preparation as the enzyme source. The present method described here, which is an application of the method (II) to CoA production, gave a higher yield of CoA under the appropriate fermentative conditions. This is considered to be very effective for the large scale production of CoA, though it requires somewhat tedious purification procedures when compared with the original dried cell method, as will be described in a subsequent section.

In general, fermentative production of nucleotides, as described above, are greatly affected by the cellular permeability of the microorganisms used. The removal of permeability barriers is given by drying the cells in the method (I), and by controlling the levels of trace nutrients or trace metals, or by the addition of reagents affecting cellular permeability such as surfactants, antibiotics, and so on, in the method (II). In the present experiment, the surfactant was one of the most important factors for the production of CoA. As described previously, formation of CoA with yeast cells was stimulated by the addition of certain cationic surfactants. The strong stimulation of CoA formation by dried cells or washed cells of *Brevibacterium ammoniagenes* IFO 12071 was also observed by the addition of some anionic- and cationic surfactants. Nara *et al.* (73) reported that the enzymes relating to ribotide synthesis were excreted into the medium upon the addition of surfactant. They reported also that transphosphorylase, catalyzing the phosphorylation of AMP to ATP, was leaked into the medium together with the above enzymes (86). These observations suggest that the CoA synthesizing systems leaked out upon the addition of surfactant, coupled with the transphosphorylase system, and then, at least partly, CoA was accumulated extracellularly.

Section V.

A New Process for the Production of Coenzyme A[e,g)]

A new process has been described for the preparation of CoA of high purity from the cultured broth of *Brevibacterium ammoniagenes* IFO 12071. The product was obtained in a high yield by the use of Duolite S-30, charcoal, and Dowex 1 x 2, and identified chemically and enzymatically. The method is simple, rapid, and compact, requires no special equipment, and has been shown to be adaptable for preparing large amounts of highly pure CoA.

INTRODUCTION

The author has previously described the isolation of CoA in a high yield from a reaction mixture of dried cells of *Brevibacterium ammoniagenes* IFO 12071 by the use of charcoal and DEAE-cellulose (see Section I). Further, it was found that the organism accumulated 2-5.5 mg/ml of CoA in the culture broth (see Section III and IV).

The present section deals with the isolation and identification of CoA with the objective of large scale production.

EXPERIMENTAL AND RESULTS

Materials

The microorganism used was *Brevibacterium ammoniagenes* IFO 12071. DEAE-cellulose (0.9 meq/g) was provided from Green Cross Corporation, Osaka. Phosphotransacetylase from *Clostridium kluyveri* was purchased from Boehringer, Mannheim. Alkaline phosphatase from calf intestinal mucosa and phosphodiesterase from *Crotalus adamanteus* were purchased from Sigma Chemical Co., St. Louis. Other materials and chemicals were obtained from commercial sources.

Analytical methods

CoA was assayed by the phosphotransacetylase method of Stadtman *et al.* (43) using the enzyme of *Escherichia coli* Crookes AKU 0001, and also by the method of Bergmeyer *et al.* (44) for the characterization of isolated products. Total pantothenate was measured by the method of Novelli (47). Adenine content was determined by ultraviolet absorption in 0.1 M HCl using E_{260}=15000 as the standard value. Phosphorus was measured by the method of Allen (87) and sulfhydryl by the method of Ellman (49). Paper chromatography was carried out by the ascending technique on Toyo Roshi No.53 paper. The solvent systems used were I, isobutyric acid-0.5 N ammonium hydroxide-0.1 M EDTA (100:60:1.6); II, ethanol-1 M ammonium acetate, pH 7.5 (5:2). Paper electrophoresis was carried out on Toyo Roshi No.53 paper impregnated with 0.2 M acetate buffer, pH 3.5, at 35 mA/15 cm (4 KV) for 30 min. Adenine-containing compounds were located with a UV-lamp. Phosphorus-containing compounds were located with Hanes and Isherwood spray (50) followed by ultraviolet irradiation; sulfhydryl and disulfide compounds were detected with Toennies and Kolb spray (51).

Process

1) Cultivation. *Brevibacterium ammoniagenes* IFO 12071 was cultivated aerobically for 5 days at 28°C. The details of cultivation have been described in the preceding section. The culture filtrate (about 1000 ml) contained about 2.5 g of CoA as the disulfide (or mixed disulfides) and some intermediates of CoA biosynthesis, such as 4'-phosphopantothenic acid and 4'-phosphopantetheine (disulfide or mixed disulfide), in high concentrations.

2) Purification. Initially the usual biochemical laboratory method was employed. The culture filtrate (50 ml) was applied to a column of charcoal (4 x 4 cm), and the substances adsorbed were eluted with 40% acetone containing

0.028% ammonia. CoA-containing fractions were well separated from brown materials (see Fig. 1, (B)). Appropriate fractions containing CoA were combined, concentrated to a small volume (50 ml) under reduced pressure, and 2-mercaptoethanol (17 ml) was added. The mixture was left for 24 hr at 10°C, and then it was applied to a column of DEAE-cellulose (chloride form, 3 x 18 cm). Elution with 0.02 M LiCl in 0.003 M HCl (1000 ml) removed AMP, 4'-phosphopantothenic acid, 4'-phosphopantetheine, and ADP. Then further elution was carried out by a 4000 ml linear salt gradient (0.03-0.1 M LiCl in 0.003 M HCl). For both the initial and gradient elutions 2-mercaptoethanol was present in the eluting solution at a concentration of 0.1%. Two major ultraviolet-absorbing peaks were separated. The first peak (CoASH) was adjusted to pH 4.5 with LiOH, and evaporated to dryness. The residue was dissolved in a small volume of methanol and the lithium salt of CoASH was precipitated by the addition of 20 volumes of acetone. The salt was collected, and washed repeatedly with methanol-acetone (1:9). After drying over P_2O_5 *in vacuo*, the trilithium salt of CoASH was obtained as a fine white powder (40 mg). The product was homogeneous with respect to ultraviolet absorption, sulfhydryl, and phosphorus, when chromatographed on paper. Venom phosphodiesterase digestion gave spots identical with 3',5'-ADP and 4'-phosphopantetheine.* *Anal.* Calcd. for $C_{21}H_{33}O_{16}N_7P_3SLi_3 \cdot 8H_2O$: C,27.13; H,5.37; N, 10.54%. Adenosine : phosphorus : sulfhydryl : pantothenic acid, 1:3:1:1. Found: C,27.01; H,5.01; N,10.30%. Adenosine : phosphorus : sulfhydryl : pantothenic acid, 1:3.03:1.00:0.99. UV λmax in 0.1 M HCl, 259 nm; UV λmax in 0.1 M NaOH, 260 nm. Assayed by the phosphotransacetylase method of Bergmeyer *et al.* (44), the product showed a 101% activity on the basis of its adenosine content.

* This compound was prepared by the method as described in a subsequent section.

The second peak (ATP) was worked up as described above to give a lithium salt of ATP (71.5 mg). The product was homogeneous with respect to ultraviolet absorption and phosphorus when chromatographed on paper. The molar ratio of adenosine : phosphorus was 1:3.10 (Required, 1:3). UV λmax in 0.1 M HCl, 259 nm; UV λmax in 0.1 M NaOH, 260 nm.

Next, a large scale purification was carried out with the objective of application to industrial production. The culture filtrate (1000 ml) was passed

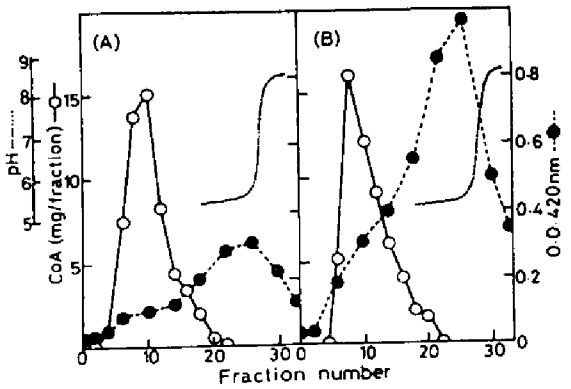

FIG. 1. Separation of CoA and Brown Materials on a Charcoal Column.

Fifty ml of the culture filtrate were applied on a column (4 x 4 cm) of charcoal after passing through a column (2 x 5 cm) of Duolite S-30 (A). (B) is a control run (without Duolite S-30). Each fraction contains 10 ml of eluate.

through a column of Duolite S-30 (3 x 20 cm) to remove brown materials, and the column was washed with water (100 ml). The solution passing through the column was directly applied to a column of charcoal (5.5 x 30 cm), and the substances adsorbed were eluted with 40% acetone containing 0.028% ammonia (A result of a small scale experiment to know the effect of Duolite S-30 and charcoal on the removal of brown materials is shown in Fig. 1.). The eluted solution containing acetone was directly adsorbed to a column of Dowex 1 x 2 (chloride form, 5.5 x 42.5 cm). The products were eluted from the column with solutions of LiCl in HCl. An example of the elution pattern is shown in Fig. 2. In each case, appropriate

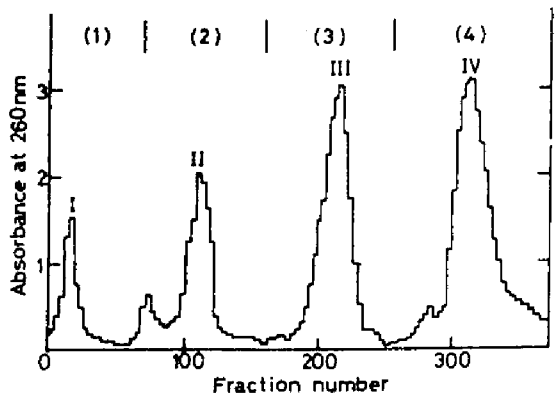

FIG. 2. A Typical Elution Pattern of Nucleotides in Culture Broth from a Dowex 1x2 Column.

Nucleotides were eluted from the column (chloride form, 2.5 x 25 cm) with 0.01 M HCl (1), 0.04 M LiCl in 0.01 M HCl (2), 0.2 M LiCl in 0.01 M HCl (3), and 0.4 M LiCl in 0.01 M HCl (4). Each fraction contains 10 ml of eluate. Peak I, AMP; Peak II, ADP; Peak III, ATP; Peak IV, CoASSCoA.

fractions were combined, adjusted to pH 4.5 with LiOH, then concentrated to a small volume, and the lithium salt was isolated in the usual way. Yields were as follows: AMP, 127 mg (Found: ratio of adenosine : phosphorus, 1:1.05; Required: 1:1). ADP, 604 mg (Found: ratio of adenosine : phosphorus, 1:2.15; Required: 1:2). ATP, 1212 mg (Found: ratio of adenosine : phosphorus, 1:3.11; Required: 1:3). CoASSCoA, 1007 mg (Found: ratio of adenosine : phosphorus: pantothenic acid, 1:3.05:0.90; Required: 1:3:1). This disulfide contained a trace of a contaminating ultraviolet-absorbing compound (*Rf* 0.19 in solvent I), when chromatographed on paper. Assayed by the phosphotransacetylase method of Stadtman *et al.* (43) against purified CoA (92% by weight) standard, it showed 83-87% activity.

For the purpose of reduction, the above disulfide (250 mg) was dissolved in water (5 ml) and 2-mercaptoethanol (5 ml) was added. The mixture was left for 24 hr at 10°C, adjusted to pH 4.5 with LiOH, and worked up as described above to give a lithium salt (222 mg, Found: ratio of adenosine : phosphorus : sulfhydryl : pantothenic acid, 1:3.07: 0.88:0.93; Required: 1:3:1:1), which

TABLE I. PAPER CHROMATOGRAPHY AND PAPER ELECTROPHORESIS

Compound	*Rf* I	*Rf* II	Electrophoresis*
CoASH	0.57	0.16	18.5
CoASSCoA	0.38	0.01	19.5
AMP	0.55	0.13	9.7
ADP	0.44	0.04	16.5
ATP	0.33	0.02	21.0

* Migration towards the anode is given in cm.

was chromatographically shown to contain only CoASH and a little of the disulfide. Assayed by the phosphotransacetylase method of Bergmeyer *et al.* (44), it showed an 84% activity on the basis of its adenosine content.

A further purified sample was obtained as the thiol form from the above disulfide by reduction with 2-mercaptoethanol followed by Dowex 1 x 2 (chloride form) column chromatography. The CoASH was eluted with 0.2 M LiCl in 0.01 M HCl containing 0.1% 2-mercaptoethanol. The product was homogeneous with respect to ultraviolet absorption, phosphorus, and sulfhydryl on paper chromatogram. Assayed by the phosphotransacetylase method of Bergmeyer *et al.* (44), it now showed a 97% activity on the basis of its adenosine content.

DISCUSSION

CoA has been prepared by extraction from microorganisms (20,21,25-27,29) and by chemical synthesis (28,30-32). However, these methods are not practical because of their lower yield or their intricacy. A new improved process with a microorganism has now been developed. Incubation of pantothenic acid, cysteine, and ATP with the dried cells of *Brevibacterium ammonigenes* IFO 12071 gave CoA in a high yield. The product was isolated as the thiol form at a high level of purity by the use of charcoal and DEAE-cellulose (see Section I). Further, cultivation of the organism with pantothenic acid, cysteine, AMP, and a surfactant gave a higher accumulation of CoA (2-5.5 mg/ml) in the culture broth (see Section IV).

The product in the culture broth was purified by the use of Duolite S-30, charcoal, and Dowex 1 x 2, and isolated as the lithium salt in good yield.

As described in Section I, the product was directly isolated as the thiol form from the reaction mixture without prior separation of ATP, since the ATP in the reaction mixture was completely consumed for the CoA formation. On the other hand, in the present process it was necessary to remove ATP in order to obtain the product in high yield and high purity. Isolation of the product directly as the disulfide form rather than the thiol form brought a complete separation of ATP, and led to a considerably improved yield. The disulfide was readily converted to the thiol form by treatment with 2-mercaptoethanol. A further improved yield of CoA might be attained by recovering the mixed disulfides of CoA which might be present in the culture broth.

The present process is simple, rapid, and compact, and requires no special equipment. From a practical point of view, it possesses considerable advantages over any other microbial process previously reported (20,21,25-27,29) in that the product can be purified in a higher yield with a compact plant, without complex purification procedures. This not only speeds up purification and isolation but also gives a highly purified product. Therefore, the present process would be suitable for industrial production.

A further practical point is that AMP added as a precursor for CoA formation, can be recovered as ATP or ADP and the isolation of some intermediates of CoA biosynthesis such as 4'-phosphopantothenic acid and 4'-phosphopantetheine is possible if necessary. In a similar process, these intermediates were obtained in higher yields, which will be described in a subsequent section.

Section VI.

Microbial Synthesis of Intermediates of Coenzyme A Biosynthesis[e,h)]

Greater production of 4'-phosphopantothenic acid and 4'-phosphopantetheine by a microorganism were described. The incubation of pantothenic acid and ATP with washed cells of *Brevibacterium ammoniagenes* IFO 12071 gave 4'-phosphopantothenic acid in a high yield. Cultivation of the organism with pantothenic acid and AMP also gave 4'-phosphopantothenic acid in a high yield. In a similar fashon, 4'-phosphopantetheine was readily obtained in a good yield. The products were identified chemically and enzymatically.

INTRODUCTION

Phosphorylation of pantothenic acid was first found by Pierpoint *et al.* (75), and then Brown (14) demonstrated that 4'-phosphopantothenic acid is the first metabolite in the biosynthesis of CoA from pantothenic acid. Brown (14) also demonstrated that 4'-phosphopantetheine, the third intermediate from pantothenic acid to CoA, is synthesized through a coupling of 4'-phosphopantothenic acid and cysteine, followed by decarboxylation of the cysteine moiety. As a protein-bound prosthetic group, 4'-phosphopantetheine plays a central role in the biosynthesis of fatty acids (88) and of peptides such as gramicidin S and tyrocidine (89). These important compounds (or coenzymes) have been exclusively prepared by complex chemical syntheses (28,90-93).

In the previous sections, the author has demonstrated that CoA and some of its biosynthetic intermediates can be synthesized in higher yields by a microbial process. The optimum conditions for CoA production and the purification and isolation of the products were also described.

This section deals with a new preparative method for the intermediates of CoA biosynthesis.

MATERIALS AND METHODS

Chemicals. ATP was kindly given by Kyowa Hakko Kogyo Co., Ltd., Tokyo. Authentic samples of 4'-phosphopantothenic acid, 4'-phosphopantothenoylcysteine, and 4'-phosphopantetheine (disulfide form) used for paper chromatography and paper electrophoresis were the kind gifts of Dr. M. Shimizu, Daiichi Seiyaku Co., Ltd., Tokyo. DEAE-cellulose (0.9 meq/g) was provided by Green Cross Corporation, Osaka. Alkaline phosphatase from calf intestinal mucosa and phosphodiesterase from *Crotalus adamanteus* were obtained from Sigma Chemical Co., St. Louis. Other chemicals were obtained from commercial sources.

Microorganism and cultivation. *Brevibacterium ammoniagenes* IFO 12071 was used. The media for the microorganism and cultivation techniques were the same as those described in the previous sections.

Analytical methods. Pantothenic acid was measured microbiologically using *Saccharomyces carlsbergensis* ATCC 9080 (46) and *Lactobacillus plantarum* ATCC 8014 (45). 4'-Phosphopantothenic acid was measured by following the disappearance of pantothenic acid in the reaction mixture or culture broth. This was checked by measuring the recovery of pantothenic acid after digestion with alkaline phosphatase. CoA was measured by the phosphotransacetylase method of Stadtman *et al.* (43) using the enzyme of *Escherichia coli* Crookes AKU 0001 as described in Section I. Total pantothenate was measured by the method of Novelli (47). The amount of the intermediates of CoA biosynthesis (4'-phosphopantothenoylcysteine, 4'-

phosphopantetheine, and 3'-dephospho-CoA) or 4'-phosphopantetheine was calculated by subtracting pantothenic acid, 4'-phosphopantothenic acid, and CoA from total pantothenate. In some cases, 4'-phosphopantetheine was also measured as pantetheine with *Lactobacillus bulgaricus* Bl after digesting the sample with alkaline phosphatase (94). *Lactobacillus bulgaricus* Bl was a kind gift of Dr. M. Shimizu, Daiichi Seiyaku Co., Ltd., Tokyo. Other assays were the same as those described in Section I.

RESULTS

Formation of the intermediates of CoA biosynthesis

In order to investigate whether the intermediates of CoA biosynthesis are formed from pantothenic acid or 4'-phosphopantothenic acid, several reaction systems were tested as shown in Table I. In the complete reaction mixture, CoA was formed, as has been already pointed out in the previous sections. When cysteine was omitted from the complete reaction mixture, pantothenic acid completely disappeared, without the formation of CoA. It was completely recovered by treatment with alkaline phosphatase, suggesting the presence of the phosphorylated form of pantothenic acid in the reaction mixture. When pantothenic acid and ATP were respectively replaced by 4'-phosphopantothenic acid and CTP, 4'-phosphopantothenic acid disappeared without any detectable formation of CoA. On the paper chromatogram a sulfhydryl-containing spot, different from CoA and cysteine, was detected at *Rf* 0.65 using a solvent of *n*-butanol-acetic acid-water (5:2:3, by vol.). It was recovered as pantothenic acid by digestion with a mixture of alkaline phosphatase and hog kidney amidase (47), suggesting the presence of a conjugated form of pantothenic acid and cysteine in the reaction mixture.

TABLE I. FORMATION OF INTERMEDIATES OF CoA BIOSYNTHESIS

The complete reaction mixture contained, in 1 ml, 2 μmoles of sodium pantothenate, 4 μmoles of cysteine, 10 μmoles of ATP, 10 μmoles of $MgSO_4$, 150 μmoles of potassium phosphate buffer, pH 6.5, 1 mg of sodium laurylsulfate, and 100 mg of dried cells of *Brevibacterium ammoniagenes*. The reactions were carried out at 37°C for 6 hr, and terminated by immersing the tube in boiling water for 5 min. After adding 3 ml of water, cells were removed by centrifugation. The supernatant was employed in the determination of the products.

Reaction mixture	Compound found (μmoles)		
	Pantothenic acid		CoA
	before Pase* digestion	after Pase digestion	
Complete	0.00	0.09	1.30
Without cysteine	0.00	2.01	0.01
With CTP replacing ATP	1.40	1.40	0.01
With 4'-phosphopantothenic acid replacing pantothenic acid	0.00	0.00	1.56
With 4'-phosphopantothenic acid and CTP replacing pantothenic acid and ATP	0.00	0.00	0.01

* Pase: alkaline phosphatase.

Isolation and identification of the intermediates of CoA biosynthesis

i) 4'-Phosphopantothenic acid. a) The reaction mixture (340 ml) containing 3.4 mmoles of sodium pantothenate,

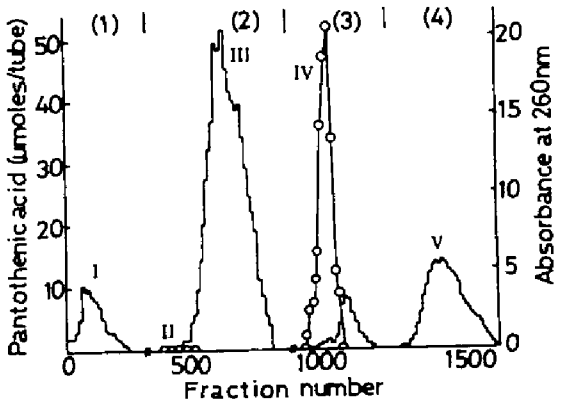

FIG. 1. Chromatography of the Products in the Reaction Mixture on a Dowex 1x2 Column.

Compounds were eluted with water (1), 0.007 M HCl (2), 0.01 M LiCl in 0.01 M HCl (3), and 0.03 M LiCl in 0.01 M HCl (4). Each fraction contains 20 ml of eluate. Peak I, adenine and adenosine; Peak II, free pantothenic acid; Peak III, AMP; Peak IV, 4'-phosphopantothenic acid; Peak V, ADP.

5.1 mmoles of ATP, 3.4 mmoles of $MgSO_4$, 34 mmoles of potassium phosphate buffer, pH 6.0, 680 mg of sodium laurylsulfate, and washed cells of *Brevibacterium ammoniagenes* (20.4 g) was incubated for 8 hr at 37°C. Then, the mixture was immersed for 10 min in a boiling water bath and the cells were removed by centrifugation. The supernatant was diluted to 1000 ml with water and applied to a column of Dowex 1 x 2 (chloride form, 4.2 x 40 cm). The elution was carried out as shown in Fig. 1. Elution with 0.01 M LiCl in 0.01 M HCl gave a peak of phosphorylated pantothenic acid. Appropriate fractions were combined, adjusted to pH 4.5 with LiOH, and evaporated under reduced pressure below 40°C. The final residue was dissolved in a small volume of methanol and the lithium salt was precipitated by the addition of 20 volumes of acetone. The salt was collected, washed repeatedly with methanol-acetone (1:10, by vol.), and dried over P_2O_5 *in vacuo* (yield, 608 mg; purity, based on pantothenic acid content, 84-88%). For the purpose of further purification, a portion of the above lithium salt (60 mg) was dissolved in water (10 ml) and applied to a column of DEAE-cellulose (chloride form, 2 x 30 cm). Elution was carried out by a 1100 ml linear salt gradient (0-0.05 M LiCl in 0.003 M HCl). Appropriate fractions were combined, and passed through a column of Amberlite IR-120 (H^+ form). The eluate and washings were neutralized with $Ba(OH)_2$ and evaporated to dryness. The residue was dissolved in a small volume of methanol, clarified, and poured into 20 volumes of acetone to give a white precipitate. This was collected, washed, and then dried (yield, 34 mg). The product was homogeneous with respect to phosphorus and pantothenic acid on paper chromatogram. Phosphatase digestion gave spots identical with inorganic phosphate and pantothenic acid. It was partially active in enhancing the growth of *Saccharomyces carlsbergensis* ATCC 9080 and *Lactobacillus plantarum* ATCC 8014 at higher levels and became fully active after phosphatase digestion. IR νmax in KBr pellet, 3300,2955,2880,1648,1567, 1410,1365,1315,1260,1120,1085,977,805 cm^{-1}. The NMR spectrum of the lithium

TABLE II. PAPER CHROMATOGRAPHY AND PAPER ELECTROPHORESIS

Ascending paper chromatography was performed on Toyo Roshi No. 53 paper using solvent I, *n*-butanol-acetic acid-water (5:2:3, by vol.); solvent II, isobutyric acid-0.5 N ammonium hydroxide-0.1 M EDTA (100:60:1.6, by vol.).

Paper electrophoresis was performed on Toyo Roshi No. 53 paper for 30 min (A), or 50 min (B) at 30 mA/15 cm (4 KV) using A, 0.05 M acetate buffer, pH 3.5; B, 0.05 M borate buffer, pH 9.4. Migration towards the anode is given in cm.

Compound	*Rf*		Migration	
	I	II	A	B
4'-Phosphopantothenic acid	0.59	0.53	12.0	22.3
4'-Phosphopantetheine (thiol form)	0.65	0.66	4.3	15.5
4'-Phosphopantetheine (disulfide form)	0.40	-*	9.6	14.4
Pantothenic acid	0.79	0.73	-	-
CoA	0.24	0.57	13.5	16.1

* not tested.

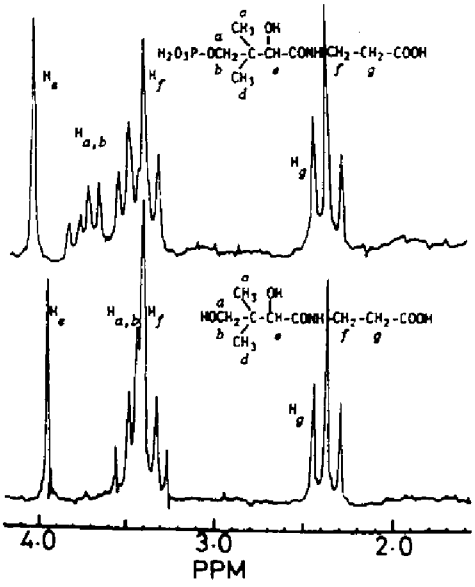

FIG. 2. Comparative NMR Spectra of 4'-Phosphopantothenic Acid and Pantothenic Acid.

NMR spectra were measured on a Hitachi Perkin-Elmer R-22 spectrometer at 90 MHz in D_2O with DSS as the internal standard. Top, 4'-phosphopantothenic acid; bottom, pantothenic acid.

salt (84-88% pure) is shown in Fig. 2. The splitting pattern of the sample is consistent with that of pantothenic acid except that the signals of the 4'-C-protons are shifted to a lower field than those of pantothenic acid and re-splitted by coupling with phosphorus. *Anal.* Calcd. for $C_9H_{15}O_8NPBa_{1.5}\cdot 3H_2O$: C,19.43; H,3.80; N,2.52%. Phosphorus : pantothenic acid, 1:1. Found: C,19.79; H,3.52; N,2.48%. Phosphorus : pantothenic acid, 1:0.97.

b) *Brevibacterium ammoniagenes* was cultivated aerobically at 28°C in a medium composed of 10 g of glucose, 1 g of peptone, 2 g of K_2HPO_4, 1 g of $MgSO_4\cdot 7H_2O$, 0.6 g of urea, 0.2 g of AMP, and 100 ml of water, pH 7.6. After 3 days of cultivation, 0.2 g of calcium pantothenate and 0.1 g of cetylpyridinium chloride were added, then the cultivation was continued for a further 2 days. The details of cultivation were described in Section IV. The cultured broth (60 ml), containing about 150 mg of phosphorylated pantothenic acid, was boiled for 3 min and centrifuged. The supernatant, after being treated with charcoal as described in Section I, was applied to a column of Dowex 1 x 2 (chloride form, 2.5 x 25 cm). Elutions with 0.01 M LiCl in 0.01 M HCl, 0.03 M LiCl in 0.01 M HCl, and 0.2 M of LiCl in 0.01 M HCl gave phosphorylated pantothenic acid, ADP, and ATP, respectively. Each fraction was worked up as described above to give the lithium salt of the product. Finally, the lithium salt of 4'-phosphopantothenic acid was obtained as a yellowish white powder (yield, 103 mg; purity, based on pantothenic acid content, 81-85%). Yields of ADP and ATP as by-products were 60 mg and 102 mg. The lithium salt of 4'-phosphopantothenic acid obtained was further purified. A portion of the lithium salt (80 mg) was dissolved in water (12 ml) and applied to a column of DEAE-cellulose (chloride form, 2 x 30 cm). Elution was carried out by a 1100 ml linear salt gradient (0-0.05 M LiCl in 0.003 M HCl), and the trilithium salt was obtained (yield, 48 mg; *Anal.* Calcd. for $C_9H_{15}O_8NPLi_3\cdot 4H_2O$: C,27.78; H,5.96; N,3.60%. Phosphorus : pantothenic acid, 1:1. Found: C,27.49; H,6.00; N,3.62%. Phosphorus : pantothenic acid, 1:1.04).

ii) 4'-Phosphopantetheine. a) The reaction mixture (20 ml) containing 150 μmoles of 4'-phosphopantothenic acid (lithium salt prepared by the above method), 300 μmoles of cysteine, 300 μmoles of CTP, 200 μmoles of $MgSO_4$, 3 mmoles of potassium phosphate buffer, pH 6.5, 40 mg of sodium laurylsulfate, and washed cells of *Brevibacterium ammoniagenes* (1.2 g) was incubated for 5 hr at 37°C. Then, the mixture was worked up as described above. The resultant supernatant was diluted to 100 ml with water and applied to a column of Dowex 1 x 2 (chloride form, 2 x 20 cm), and the column was washed with water (400 ml). Elution with 0.005 M HCl removed CMP. 4'-Phosphopantetheine was eluted together with CDP with 0.01 M LiCl in 0.01 M HCl. Appropriate fractions were combined, adjusted to pH 5, and evaporated to dryness. The residue was dissolved in a small volume of methanol and insoluble materials were filtered out. The clear solution was treated with Amberlite IR-120 (H^+ form) and the acidic eluate was neutralized with $Ca(OH)_2$. This was applied to a column of DEAE-cellulose (chloride form, 2 x 30 cm). Elution was carried out by a 1100 ml linear salt gradient (0-0.03 M $CaCl_2$ in 0.003 M HCl). For both initial and gradient elutions, the eluting solution contained 0.1% 2-mercaptoethanol. 4'-Phosphopantetheine was well separated from the remaining CDP. Appropriate fractions were combined, neutralized with $Ca(OH)_2$, concentrated to a small volume, clarified, and then evaporated to dryness. The residue was dissolved in a small volume of ethanol. Addition of 15 volumes of ether gave the calcium salt of 4'-phosphopantetheine. The salt was collected, washed, and then dried (yield, 41 mg). The product was homogeneous with respect to phosphorus and sulfhydryl on paper chromatogram. Its *Rf* value was the same as that of the product of CoA digestion with venom phosphodiesterase. Treat-

ment with H_2O_2 gave the disulfide, which showed an *Rf* value identical to that of an authentic 4'-phosphopantetheine (disulfide form). Hydrolysis of the isolated sample with alkaline phosphatase gave a sulfhydryl compound (*Rf* 0.84 in a solvent system of *n*-butanol-acetic acid-water (5:2:3, by vol.)) and inorganic phosphate. Treatment of the digestion product with H_2O_2 gave a disulfide which was identical with an authentic pantethine (*Rf* 0.81 in a solvent system of *n*-butanol-acetic acid-water (5:2:3, by vol.)). The isolate was partially active in enhancing the growth of *Lactobacillus bulgaricus* B1 without phosphatase digestion, and released the theoretical amount of pantetheine after phosphatase digestion. The NMR spectrum is shown in Fig. 3. The resemblance of the splitting pattern to that of 4'-phosphopantothenic acid suggests that phosphorus attaches to the 4'-hydroxyl group in the pantothenoyl moiety. *Anal.* Calcd. for $C_{11}H_{21}O_7N_2SP$-$Ca \cdot 3H_2O$: C,29.33; H,6.04; N,6.22%. Phosphorus : pantothenic acid : sulfhydryl, 1:1:1. Found: C,28.94; H,5.99; N,6.22%. Phosphorus : pantothenic acid : sulfhydryl, 1:0.98:0.92.

b) *Brevibacterium ammoniagenes* was cultivated in the same medium as described above. (In this case, AMP was omitted.). After 3 days of cultivation, 0.2 g of calcium pantothenate, 0.2 g of cysteine, 0.15 g of CMP, 0.15 g of UMP, and 0.1 g of cetylpyridinium chloride were added to the culture (100 ml). Then the culture was continued for a further 2 days. The cultured broth (100 ml) was worked up as described above. The supernatant was applied to a column of charcoal (4 x 8 cm), and the adsorbed substances were eluted with 40% acetone containing 0.028% ammonia. The acidic eluate was concentrated to 50 ml. Twenty ml of 2-mercaptoethanol were added to the concentrate, which was then left at 10°C overnight. The mixture was adjusted to pH 7.5 with $Ca(OH)_2$, diluted to 200 ml, and applied to a column of DEAE-cellulose (chloride form, 2.5 x 30 cm). Elution was carried out by a 5000 ml linear salt gradient

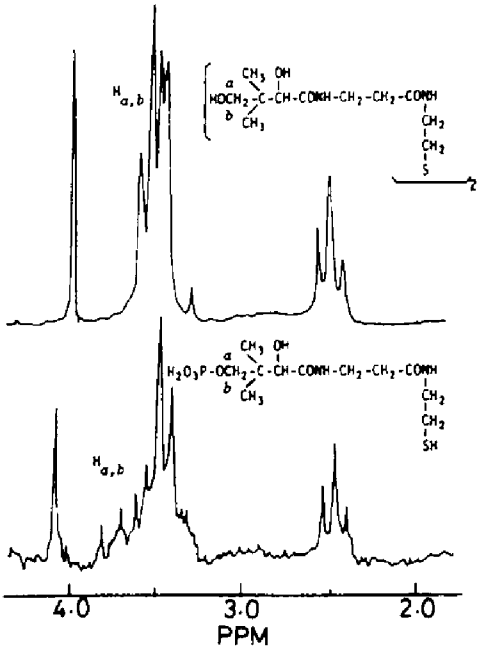

FIG. 3. Comparative NMR Spectra of 4'-Phosphopantetheine and Pantethine.

NMR spectra were measured on a Hitach Perkin-Elmer R-22 spectrometer at 90 MHz in D_2O with DSS as the internal standard. Top, pantethine; bottom, 4'-phosphopantetheine.

(0-0.03 M $CaCl_2$ in 0.003 M HCl containing 0.1% 2-mercaptoethanol). Appropriate fractions were combined, and worked up as described above to give the calcium salt of 4'-phosphopantetheine (yield, 135 mg; purity, based on pantothenic acid content, 74-80%). This was further purified by a DEAE-cellulose column (chloride form, 2 x 35 cm) chromatography using a 2000 ml linear salt gradient (0-0.03 M $CaCl_2$ in 0.003 M HCl containing 0.1% 2-mercaptoethanol). Then finally 4'-phosphopantetheine was obtained as the calcium salt (yield, 83 mg; *Anal.* Calcd. for $C_{11}H_{21}O_7N_2SPCa \cdot 3H_2O$: C,29.33; H,6.04; N,6.22%. Phosphorus : pantothenic acid : sulfhydryl, 1:1:1. Found: C,29.57; H,5.89; N,6.42%. Phosphorus : pantothenic acid : sulfhydryl, 1:1.05:0.98).

The mechanism of the accumulation of the intermediates of CoA biosynthesis will be discussed in following sections.

Section VII.

Microbial Formations of the Intermediates of Coenzyme A Biosynthesis and their Control by Nucleotides[1)]

Microbial formation of 4'-phosphopantothenic acid, 4'-phosphopantetheine, and 3'-dephospho-CoA were described in connection with a higher accumulation of CoA.

Air dried cells of *Brevibacterium ammoniagenes* IFO 12071 phosphorylated pantothenic acid in the presence of ATP. ATP could be substituted for, partly, by ITP, GTP, ADP, UTP, and CTP. 4'-phosphopantetheine was converted to CoA only when incubated with ATP. All the other nucleotides tested appeared to lack the ability to couple with 4'-phosphopantetheine. 4'-Phosphopantetheine, but not CoA, was accumulated selectively from pantothenic acid, cysteine, and a nucleotide triphosphate other than ATP. It was also accumulated when pantethine was incubated with ITP, GTP, or UTP. In a similar fashion, 4'-phosphopantothenic acid and 4'-phosphopantetheine were able to be accumulated in the culture broth of *Brevibacterium ammoniagenes*.

3'-Dephospho-CoA was synthesized by treating the reaction mixture which had accumulated CoA with 3'-nucleotidase of *Bacillus subtilis* IFO 3032.

Nucleotide specificity of 4'-phosphopantothenoylcysteine synthetase, and that of 3'-dephospho-CoA kinase were investigated using dried cells of *Brevibacterium ammoniagenes*.

INTRODUCTION

In the preceding sections, microbial formations of intermediates of CoA synthesis from pantothenic acid with higher yields were described. They were isolated and identified as 4'-phosphopantothenic acid and 4'-phosphopantetheine.

This section describes some factors affecting the selective formation of the intermediates of CoA biosynthesis and their control by nucleotides in connection with a higher formation of CoA.

MATERIALS AND METHODS

Chemicals. 4'-Phosphopantothenic acid, 4'-phosphopantetheine, 3'-dephospho-CoA, and CoA were synthesized microbiologically as described in the previous sections. ATP, ADP, GTP, GDP, CTP, and UTP were gifts of Kyowa Hakko Kogyo Co., Ltd., Tokyo. DEAE-cellulose (0.9 meq/g) was provided by Green Cross Corporation, Osaka. CDP was purchased from Seikagaku Kogyo Co., Ltd., Tokyo. Pantethine and intestinal alkaline phosphatase (Type I and Type IV) from calf mucosa were obtained from Sigma Chemical Co., St. Louis. Acetylphosphate was prepared by the method of Avison (42). All other reagents were commercial products of an analytical grade of purity.

Microorganism and cultivation. *Brevibacterium ammoniagenes* IFO 12071 was used. The media for the bacterium and cultivation techniques have been described previously (see Section I and IV).

Preparation of dried cells. Air dried cells of the bacterium were prepared according to the method described previously (see Section I).

Assay of pantothenates. Pantothenic acid was measured microbiologically with *Saccharomyces carlsbergensis* ATCC 9080 (46). 4'-Phosphopantothenic acid was

measured by following the disappearance of pantothenic acid in the reaction mixture or culture broth. This was checked by measuring the recovery of pantothenic acid after digestion with alkaline phosphatase. CoA was measured by the phosphotransacetylase method of Stadtman *et al.* (43) using the enzyme of *Escherichia coli* Crookes AKU 0001 as described in Section I. Total pantothenate was measured by the method of Novelli (47). The amount of the intermediates of CoA biosynthesis (4'-phosphopantothenoylcysteine, 4'-phosphopantetheine, and 3'-dephospho-CoA) or 4'-phosphopantetheine was calculated by subtracting pantothenic acid, 4'-phosphopantothenic acid, and CoA from the total pantothenate. In some cases, 4'-phosphopantetheine was also measured as pantetheine with *Lactobacillus bulgaricus* B1 (94) after digesting the sample with alkaline phosphatase.

Paper chromatography. Ascending paper chromatography was performed on Toyo Roshi No.53 filter paper using solvent I, *n*-butanol-acetic acid-water (5:2:3, by vol.); solvent II, *n*-propanol-28% ammonium hydroxide-water (6:3:1, by vol.); and solvent III, isobutyric acid-0.5 N ammonium hydroxide-0.1 M EDTA (100:60:1.6, by vol.). Nucleotides were located with a UV-lamp. Phosphorus-containing compounds were located with Hanes and Isherwood spray (50) followed by ultraviolet irradiation; sulfhydryl and disulfide compounds were detected with Toennies and Kolb spray (51).

Preparation of 3'-nucleotidase. A crude 3'-nucleotidase of *Bacillus subtilis* IFO 3032 was prepared according to the method of Igarashi and Kakinuma (95).

Other methods. Adenine content was determined by ultraviolet absorption in 0.1 M HCl using E_{260} = 15,000 as a standard value. Phosphorus was measured by the method of Allen (87) and sulfhydryl by the method of Ellman (49).

RESULTS

Phosphorylation of pantothenic acid

When cysteine was omitted from the complete reaction mixture, almost all the pantothenic acid initially present was consumed without the formation of CoA. It was completely recovered by treatment with alkaline phosphatase. This phosphorylaion of pantothenic acid was dependent on ATP. ATP could be substituted for, partly, by ITP, GTP, UTP, ADP, and CTP (Table I). Similar results were also obtained when each nucleoside monophosphate as indicated in Table I and pantothenic acid were added to the culture of *Brevibacterium ammoniagenes*. The isolation and identification of 4'-phosphopantothenic acid have been described in Section VI.

TABLE I. NUCLEOTIDE REQUIREMENT FOR THE PHOSPHORYLATION OF PANTOTHENIC ACID

(A) Two μmoles of sodium pantothenate (PaA) and 10 μmoles of nucleotide as indicated were incubated at 37°C for 6 hr with 10 μmoles of $MgSO_4$, 150 μmoles of potassium phosphate buffer, pH 6.5, 1 mg of sodium laurylsulfate, and 100 mg of dried cells of *Brevibacterium ammoniagenes* in a total volume of 1 ml.

(B) *Brevibacterium ammoniagenes* was cultivated in the medium as described previously (see Section IV.) except that AMP was omitted. PaA (calcium salt), nucleotide as indicated, and cetylpyridinium chloride were added to the 3 day cultures at the concentration of 2 mg/ml, 2 mg/ml, and 1 mg/ml, respectively, and the cultivation was continued for a further 2 days.

After boiling the reaction mixture for 5 min or the cultured broth for 3 min, cells were removed by centrifugation. The supernatant was employed in the determination of PaA.

(A)		(B)	
Nucleotide added	PaA consumed (μmoles/ml)	Nucleotide added	PaA consumed (mg/ml)
ATP	2.0	AMP	2.0
ITP	1.9	IMP	1.0
GTP	1.8	GMP	2.0
UTP	1.2	UMP	1.5
CTP	0.4	CMP	0.7
ADP	1.8	None	0.5
None	0.0		

Synthesis of 4'-phopshopantetheine

Coupling of 4'-phosphopantothenic acid with cysteine. As can be seen in Table II, 4'-phosphopantothenic acid was most rapidly coupled with cysteine when CTP was added to the reaction mixture. CTP could be substituted for, partly, by CDP, ATP, and ADP, but GTP, UTP, and ITP were ineffective under the conditions tested. The reaction with CTP, CDP, or ADP gave 4'-phosphopantetheine, while CoA was the main product in the reaction mixture with ATP. Isolation and identification of 4'-phosphopantetheine have been described in Section VI.

TABLE II. NUCLEOTIDE REQUIREMENT FOR THE COUPLING OF 4'-PHOSPHOPANTOTHENIC ACID WITH CYSTEINE

The reaction mixture contained, in 1 ml, 5 μmoles of 4'-phosphopantothenic acid (P-PaA), 10 μmoles of cysteine, 15 μmoles of nucleotide as indicated, 10 μmoles of $MgSO_4$, 150 μmoles of potassium phosphate buffer, pH 6.5, 1 mg of sodium laurylsulfate, and 60 mg of dried cells of *Brevibacterium ammoniagenes*. The reaction was carried out for 3 hr at 37°C, and terminated by immersing the tube in boiling water for 5 min. After adding 3 ml of water, cells were removed by centrifugation. The supernatant was employed in the determination of the products.

Nucleotide added	P-PaA coupled (μmoles/ml)	CoA formed (μmoles/ml)
CTP	3.4	0.05
CDP	3.2	0.03
ATP	3.1	2.80
ADP	2.1	0.65
GTP	0.1	0.15
ITP	0.0	0.05
UTP	0.3	0.05
None	0.0	0.02

Coupling of 4'-phosphopantetheine with ATP. 4'-Phosphopantetheine was converted to CoA only when incubated with ATP. All other nucleotides triphosphates seemed to lack the ability to couple with this substrate (Table III).

Synthesis of 4'-phosphopantetheine from pantothenic acid and cysteine. Broad specificity for nucleotide in the phosphorylation of pantothenic acid and its restriction in the coupling of 4'-phosphopantetheine with ATP strongly suggest the possibility of synthesizing 4'-phosphopantetheine, but not CoA, from pantothenic acid and cysteine, if ATP is absent in the reaction mixture. As shown in Table IV, pantothenic acid and cysteine, when incubated with CTP and GTP, ITP, or UTP, gave 4'-phosphopantetheine selectively, while the reaction with ATP gave a high accumulation of CoA.

TABLE III. COUPLING OF 4'-PHOSPHOPANTETHEINE WITH NUCLEOTIDE

The reaction mixture contained, in 0.5 ml, 1 μmole of 4'-phosphopantetheine (P-PaSH), 5 μmoles of nucleotide as indicated, 5 μmoles of cysteine, 5 μmoles of $MgSO_4$, 75 μmoles of potassium phosphate buffer, pH 6.5, 0.5 mg of sodium laurylsulfate, and 50 mg of dried cells of *Brevibacterium ammoniagenes*. The reaction was carried out for 2 hr at 37°C, and terminated by immersing the tube in boiling water for 5 min. After adding 1.5 ml of water, cells were removed by centrifugation. The supernatant was employed in the determination of the products.

Nucleotide added	P-PaSH consumed[a] (μmoles/ml)	CoA formed (μmoles/ml)
ATP	2.0	2.03
GTP	0.5	0.16
ITP	0.4	0.15
UTP	0.2	0.05
CTP	0.0	0.03
None	0.0	0.04

a) P-PaSH was measured as pantetheine by *Lactobacillus bulgaricus* B1 (94) after digesting the mixture with alkaline phosphatase (Type IV).

Isolation of 4'-phosphopantetheine from the reaction mixture with CTP and ITP was carried out. The reaction mixture (40 ml) containing 300 μmoles of sodium pantothenate, 400 μmoles of cysteine, 600 μmoles of ITP, 400 μmoles of CTP, 400 μmoles of $MgSO_4$, 6 mmoles of potassium phosphate buffer, pH 6.5, 40 mg of sodium laurylsulfate, and 4 g of dried cells of *Brevibacterium ammoniagenes* was incubated for 8 hr at 37°C. Then, the mixture was immersed for 5 min in boiling water and the cells were removed by centrifugation. The super-

TABLE IV. NUCLEOTIDE REQUIREMENT FOR THE SYNTHESIS OF 4'-PHOSPHOPANTETHEINE FROM PANTOTHENIC ACID AND CYSTEINE

Two μmoles of sodium pantothenate (PaA), 4 μmoles of cysteine, 10 μmoles of $MgSO_4$, 150 μmoles of potassium phosphate buffer, pH 6.5, 1 mg of sodium laurylsulfate, and 100 mg of dried cells of *Brevibacterium ammoniagenes* were incubated for 6 hr at 37°C with nucleotide as indicated in a total volume of 1 ml. The reaction was terminated by immersing the tube in boiling water for 5 min. After adding 3 ml of water, cells were removed by centrifugation. The supernatant was employed in the determination of the products.

Nucleotide added[a]	Compound found (μmoles/ml)		
	PaA + P-PaA[b]	P~PaSH[c]	CoA
CTP	1.40	0.61	0.01
CTP + ATP	0.09	0.61	1.30
CTP + GTP	0.38	1.66	0.30
CTP + ITP	0.38	1.66	0.19
CTP + UTP	0.86	1.14	0.08

a) The amounts of nucleotides added were: CTP, 8 μmoles; others, 10 μmoles.
b) P-PaA: 4'-phosphopantothenic acid.
c) 4'-Phosphopantetheine (P-PaSH) was measured as pantetheine by *Lactobacillus bulgaricus* B1 (94) after digesting the mixture with alkaline phosphatase (Type I). The phosphatase used contains phosphodiesterase activity, thus it is able to hydrolyze CoA to yield pantetheine. The values presented are calculated by subtracting the amount of CoA produced from that detected as pantetheine. Therfore, the values are given as a sum of P-PaSH and 3'-dephospho-CoA.

natant was applied to a column of charcoal (1.6 x 7 cm), and the adsorbed substances were eluted with 40% acetone containing 0.028% ammonia. The acidic eluate was concentrated to about 20 ml. 2-Mercaptoethanol (10 ml) was added to the concentrate, which was then left at 10°C overnight. The mixture was adjusted to pH 7.5 with $Ca(OH)_2$, diluted to 100 ml, and applied to a column of DEAE-cellulose (chloride form, 1.8 x 25 cm). Elution was carried out by a 3000 ml linear salt gradient (0–0.03 M $CaCl_2$ in 0.003 M HCl containing 0.1% 2-mercaptoethanol). 4'-Phosphopantetheine was eluted, followed by IMP. Appropriate fractions were combined, and worked up as described previously (see Section VI) to give a white powder (yield, 45 mg; ratio of phosphorus : pantetheine : sulfhydryl, 1:0.92:0.85 (required, 1:1:1); *Rf* in solvent I, 0.66 and in solvent II, 0.30).

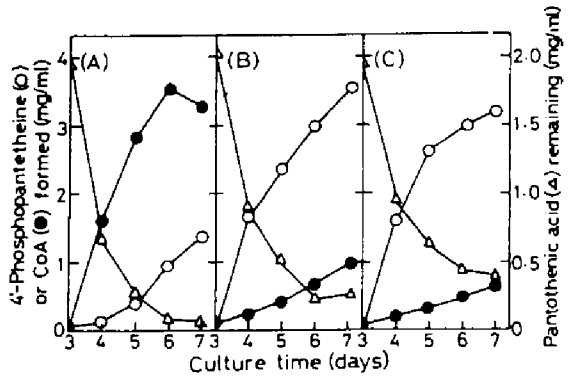

FIG. 1. Selective Accumulation of 4'-Phosphopantetheine in the Culture Broth.

Brevibacterium ammoniagenes was cultivated in the medium as described in Section IV except that AMP was omitted. To the 3 day culture, calcium pantothenate (2 mg/ml), cysteine (2 mg/ml), nucleotides (1.5 mg/ml each) as indicated, and cetylpyridinium chloride (1 mg/ml) were added, and the cultivation was continued. One ml of the broth was pipetted from the shaking flask at 24 hr intervals, boiled for 3 min, and centrifuged. The supernatant was employed in the determination of the products. The nucleotides added were CMP and AMP (A), CMP and GMP (B), or CMP and UMP (C). The amount of 4'-phosphopantetheine was calculated by subtracting the amounts of CoA, 4'-phosphopantothenic acid, and pantothenic acid from the total pantothenate. Therefore, the values are given as a sum of 4'-phosphopantetheine, 4'-phosphopantothenoylcysteine, and 3'-dephospho-CoA.

This selective synthesis of 4'-phosphopantetheine was also observed when pantothenic acid, cysteine, CMP, and GMP or UMP were added to the cultures of *Brevibacterium ammoniagenes* (Fig. 1). Isolation and characterization of the product from the culture broth was described in Section VI.

Another attempt to synthesize 4'-phosphopantetheine without the formation of CoA was tried. Incubation of pantethine with GTP, ITP, or UTP also gave 4'-phosphopantetheine, but not CoA. Isolation of the product from the reaction mixture with ITP was carried out according to a similar procedure as described above, and the product was identified as 4'-phosphopantetheine.

Synthesis of 3'-dephospho-CoA from pantothenic acid, cysteine, and ATP

Kakinuma and Igarashi (96) reported that the 3'-nucleotidase of *Bacillus subtilis* IFO 3032 attacks the 3'-position in the CoA molecule, and Kurooka *et al.* (54) applied this enzyme to prepare 3'-dephospho-CoA from commercial CoA. In order to accumulate 3'-dephospho-CoA, the reaction mixture with accumulated CoA was treated with the 3'-nucleotidase of *Bacillus subtilis* IFO 3032. The reaction mixture (40 ml) containing 300 μmoles of sodium pantothenate, 400 μmoles of cysteine, 600 μmoles of ATP, 400 μmoles of $MgSO_4$, 6 mmoles of Tris-HCl buffer, pH 7.2, 40 mg of sodium laurylsulfate, and 4 g of dried cells of *Brevibacterium ammoniagenes* was incubated for 8 hr at 37°C. Then the mixture was diluted to 80 ml, immersed for 5 min in boiling water, and the cells were removed by centrifugation. The supernatant (70 ml) containing 45 mg of CoA was combined with a solution of crude 3'-nucleotidase (30 ml), then the mixture was incubated at 37°C. The CoA initially present in the reaction mixture was inactivated gradually. After 6 hr incubation, the mixture was boiled for 5 min, centrifuged, and applied to a column of charcoal (1.6 x 7 cm). Elution, concentration, and treatment with 2-mercaptoethanol were carried out as described above. The resultant solution was adjusted to pH 7.5 with LiOH, diluted to 100 ml, and applied to a column of DEAE-cellulose (chloride form, 1.8 x 25 cm). Elution was carried out by a 4000 ml linear salt gradient (0–0.07 M LiCl in 0.003 M HCl containing 0.1% 2-mercaptoethanol). Appropriate fractions containing 3'-dephospho-CoA were combined, and worked up as described previously (see Section V), to give the lithium salt (yield, 33 mg; ratio of adenosine : phosphorus : sulfhydryl, 1:1.88:0.85, (required, 1:2:1); *Rf* in solvent III, 0.67). Elution profiles from small scale experiments before and after treatment with the 3'-nucleotidase are shown in Fig. 2.

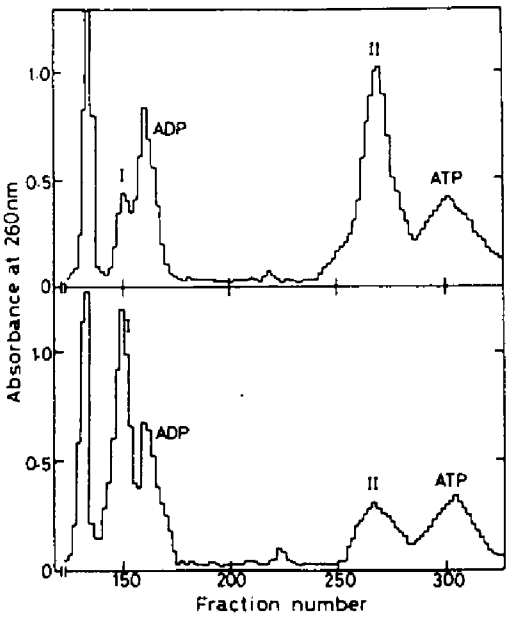

FIG. 2. Conversion of Formed CoA to 3'-Dephospho-CoA by 3'-Nucleotidase of *Bacillus subtilis* IFO 3032.

The reaction mixture containing, in 6 ml, 45 μmoles of sodium pantothenate, 60 μmoles of cysteine, 90 μmoles of ATP, 60 μmoles of $MgSO_4$, 900 μmoles of Tris-HCl buffer, pH 7.2, 6 mg of sodium laurylsulfate, and 600 mg of dried cells of *Brevibacterium ammoniagenes* was incubated for 10 hr at 37°C. The reaction was terminated by immersing the tube in boiling water for 5 min, and cells were removed by centrifugation. Two ml of the supernatant were combined with 2 ml of crude 3'-nucleotidase. The mixture was incubated for 4 hr at 37°C, boiled for 5 min, centrifuged, treated with 2 ml of 2-mercaptoethanol for 6 hr at 10°C, diluted to 80 ml with water, and applied to a column of DEAE-cellulose (chloride form, 1.6 x 60 cm). Another 2 ml of the supernatant, not treated with 3'-nucleotidase, was treated with 2 ml of 2-mercaptoethanol for 6 hr at 10°C, diluted to 80 ml, and applied to a similar column of DEAE-cellulose. Elution was carried out by a 1100 ml linear salt gradient (0–0.1 M LiCl in 0.003 M HCl containing 0.1% 2-mercaptoethanol). Fractions of 3.5 ml each were collected. Top, before treatment with 3'-nucleotidase; bottom, after treatment with 3'-nucleotidase. Peak I, 3'-dephospho-CoA; Peak II, CoA.

Formation of CoA from 3'-dephospho-CoA with various nucleotides

3'-Dephospho-CoA was converted to CoA in the presence of ATP. When ATP was replaced with ADP, GTP, UTP, or CTP,

TABLE V. FORMATION OF CoA FROM 3'-DEPHOSPHO-CoA WITH VARIOUS NUCLEOTIDES

The reaction mixture contained, in 0.5 ml, 1 μmole of 3'-dephospho-CoA, 5 μmoles of nucleotide as indicated, 5 μmoles of $MgSO_4$, 75 μmoles of potassium phosphate buffer, pH 6.5, 0.5 mg of sodium laurylsulfate, and 50 mg of *Brevibacterium ammoniagenes*. The reaction was carried out for 2 hr at 37°C, and terminated by immersing the tube in boiling water for 5 min. After adding 1.5 ml of water, cells were removed by centrifugation. The supernatant was employed in the determination of the product.

Nucleotide added	CoA formed (μmoles/ml)
ATP	2.13
ADP	1.34
GTP	1.24
ITP	1.37
UTP	0.45
CTP	0.50
None	0.07

accumulation of CoA was also observed. The results are given in Table V.

DISCUSSION

A further step in the successful process for the production of CoA has now been given: A large amount of 4'-phosphopantetheine was selectively accumulated without the formation of CoA by replacing ATP with other nucleotides. It was also produced in a higher yield when *Brevibacterium ammoniagenes* was cultivated with pantothenic acid, cysteine, CMP, and UMP (or GMP). 3'-Dephospho-CoA was obtained by converting the CoA formed by treating it with 3'-nucleotidase of *Bacillus subtilis* IFO 3032. The present process described here might be suitable not only for a biochemical laboratory method but also for further large scale production, because the process is simple and rapid, and requires no special equipment.

As will be described in the next section, an interesting property of the purified pantothenate kinase of *Brevibacterium ammoniagenes* is its partial lack of specificity with regard to the nucleotide. In the present work, experiments with dried cells and growing cells also showed this broad specificity for the nucleotide. Further, CTP and ADP, which are inactive nucleotides with the purified pantothenate kinase (see the next section), showed activity with dried cells or growing cells. On the contrary, only ATP could couple with 4'-phosphopantetheine and yield CoA. All other nucleotides tested appeared to lack the ability to couple with 4'-phosphopantetheine. The direct and exclusive accumulation of 4'-phosphopantetheine from pantothenic acid and cysteine by the bacterium may be attributed to the broad specificity for nucleotide in the phosphorylation of pantothenic acid and its restriction in the coupling of 4'-phosphopantetheine with ATP. Another route for the accumulation of 4'-phosphopantetheine, which was shown by incubating pantethine with all the nucleotides tested except ATP, may also be attributed to the facts as described above.

Now, including CoA itself, all the intermediates of CoA biosynthesis from pantothenic acid, though 4'-phosphopantothenoylcysteine is an exception, have been able to be synthesized conveniently by this microbial process.

Section VIII.

Purification and Properties of Pantothenate Kinase from *Brevibacterium ammoniagenes* IFO 12071[1)]

Pantothenate kinase (ATP : pantothenate 4'-phosphotransferase, E. C. 2.7.1.33) was purified about 200-fold from the cell extract of *Brevibacterium ammoniagenes* IFO 12071 by ammonium sulfate fractionation, DEAE-cellulose chromatography, and Sephadex G-150 gel filtration. The purified enzyme gave a single band on polyacrylamide gel electrophoresis. The molecular weight was calculated approximately 45,000. The enzyme catalyzed the formation of 4'-phosphopantothenic acid and ADP from pantothenic acid and ATP in the presence of Mg^{2+}. ATP could be substituted for, partly, by ITP, GTP, and UTP. The enzyme phosphorylated not only pantothenic acid, but also pantothenoylcysteine, pantetheine, and pantothenyl alcohol. Apparent K_m values were 6.7×10^{-5} M for pantothenic acid, 3.5×10^{-5} M for ATP, and 10^{-3} M for Mg^{2+}. The reaction was inhibited by the intermediates of CoA biosynthesis, of which CoA itself was the most effective inhibitor. Other properties of the enzyme were also investigated.

INTRODUCTION

Phosphorylation of pantothenic acid, the first step in CoA biosynthesis from pantothenic acid, has been found in microorganisms (14,75,97) and mammals (14,15,98,99). However, most of the reports published were based on experiments with crude or partially purified preparations. Difficulty in purifying this enzyme may be attributed to the fact that the enzyme is hard to stabilize (99).

In the previous sections, the author has described a novel production method for CoA from pantothenic acid, cysteine, and AMP or ATP with the bacterium *Brevibacterium ammoniagenes* IFO 12071. Now, the author has purified pantothenate kinase from *Brevibacterium ammoniagenes* as a homogeneous protein. In this section, procedures for the purification of the enzyme, and some of its characteristics are described, and its physiological significance in CoA biosynthesis by this bacterium is briefly discussed.

MATERIALS AND METHODS

Chemicals. 4'-Phosphopantothenic acid (trilithium salt), 4'-phosphopantetheine (calcium salt), 3'-dephospho-CoA (dilithium salt), and CoA (trilithium salt) were synthesized microbiologically as described in the previous sections. Pantothenoylcysteine, and 4'-phosphopantothenoylcysteine were kind gifts of Dr. M. Shimizu, Daiichi Seiyaku Co., Ltd., Tokyo. Sodium salts of ATP, ADP, GTP, GDP, ITP, UTP, NAD, NADP, and FAD were given by Kyowa Hakko Kogyo Co., Ltd., Tokyo. DEAE-cellulose (0.9 meq/g) was given by Green Cross Corporation, Osaka. Sephadex G-150 and G-200 were purchased from Pharmacia, Uppsala. Cytochrome *c* (horse heart), chymotripsinogen A (bovine pancreas), albumins (egg and bovine serum), alcohol dehydrogenases (yeast and horse liver), aldolase (rabbit muscle), catalase (beef liver), and ferritin were purchased from Boehringer, Mannheim, and alkaline phosphatase (calf mucosa) from Sigma Chemical Co., St. Louis. All other reagents were commercial products of analytical grade of purity.

Bacterial strain and growth conditions. Brevibacterium ammoniagenes IFO 12071 was used. The bacterium was cultivated at 28°C for 36 hr under shaking in a medium composed of 10 g of glucose, 15 g of peptone, 3 g of K_2HPO_4, 2 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 1 g of yeast

extract, and 1 liter of tap water, pH 7.0. The cells were harvested by centrifugation and washed with 0.01 M potassium phosphate buffer, pH 7.0. The washed cells were suspended in the same buffer at a concentration of about 25 mg/ml, and stored at -15°C. The average yield of cells was about 5 g (dry weight) per liter of the medium.

Assay of pantothenic acid. The amount of pantothenic acid was measured microbiologically using *Saccharomyces carlsbergensis* ATCC 9080 as a test organism (46).

Assay of pantothenate kinase. A mixture containing 12.5 nmoles of calcium pantothenate, 1.25 µmoles of ATP, 0.25 µmole of $MgCl_2$, 7.5 µmoles of potassium phosphate buffer, pH 6.5, and the enzyme, in a total volume of 0.25 ml was incubated at 37°C for 5-30 min. The activity of pantothenate kinase was measured as the difference between the amount of pantothenic acid initially present and that the remaining after the reaction. This was checked by measuring the recovery of pantothenic acid after digestion with intestinal alkaline phosphatase. One unit of the activity is defined as the amount of the enzyme which catalyzes the decrease of 1 nmole of pantothenic acid per min under the above conditions. Specific activity is defined as units per mg of protein.

Polyacrylamide gel electrophoresis. Electrophoresis was performed using a modified method of Ornstein and Davis (100) in 7.5% polyacrylamide gel at 4°C in Tris-glycine buffer, pH 8.3, with a current of 3 mA per tube. Instead of preparing space gel, samples dissolved in the reservoir buffer containing 10% sucrose were layered on coarse gel. The position of the protein bands was detected by staining the gel with Amido Black.

Cellulose acetate electrophoresis. Electrophoresis on cellulose acetate membrane (Carl Schleicher and Schüll, Dassel) was performed at 3 mA/3 cm (300 v) on cooling plates for 1 hr with 0.01 M potassium phosphate buffer, pH 7.0, according to the procedure of Kohn (101). The amount of protein spotted was about 80 µg (5 µl). The position of the protein bands was detected by staining the membrane with Amido Black.

Estimation of molecular weight by gel filtration. The molecular weight of pantothenate kinase was determined by Sephadex G-200 gel filtration, according to the method of Andrews (102). The column (0.9 x 30 cm) was equilibrated with 0.01 M potassium phosphate buffer, pH 7.0. The elution of the proteins was performed, using the same buffer, at a flow rate of 1 drop per 4-5 min. The proteins used as standards were used in various combinations, usually two at a time, to calibrate the column. The amounts employed for each protein were about 0.1-0.3 mg in a total volume of 0.2 ml. Elution volume for both yeast and liver alcohol dehydrogenase, catalase, and aldolase were obtained by measurement of their catalytic activities as described by Racker (103), Chance and Maehly (104), and Taylor (105), respectively, while those for both egg and serum albumins, chymotrypsinogen A, ferritin, and cytochrome *c* were determined from the absorption measurement at 280 nm or 412 nm.

Stoichiometry. A reaction mixture containing 110 nmoles of calcium pantothenate, 0.19 µmole of ATP, 0.25 µmole of $MgCl_2$, 7.5 µmoles of potassium phosphate buffer, pH 6.5, and 215 µg of the purified enzyme (specific activity, 31 nmoles/mg/min) in a total volume of 0.25 ml was incubated for 30 min at 37°C. A mixture omitting the enzyme was taken as a control run. The reaction was stopped by boiling the tube for 1 min and 50 µl of the mixture was spotted on Toyo Roshi No.53 filter paper. Compounds were separated by paper chromatography, using as solvent isobutyric acid-0.5 N ammonium hydroxide (5:3, by vol.), and each spot corresponding to ATP and ADP was eluted with 5 ml of 0.1 M HCl. The amounts of ATP and ADP were measured by the absorbance at 260 nm.

Identification of reaction products. Paper chromatography for the identifi-

cation of reaction products was performed using the following solvents: I, *n*-butanol-acetic acid-water (5:2:3, by vol.); and II, isobutyric acid-0.5 N ammonium hydroxide (5:3, by vol.). ATP and ADP were located with a UV-lamp. Phosphorus-containing compounds were located with Hanes and Isherwood spray (50) followed by ultraviolet irradiation; sulfhydryl compounds were detected with Toennies and Kolb spray (51). For special purposes, pantothenate-containing compounds were detected by the use of bioautographic techniques.

Assay of phosphatases. Phosphatase activities in the kinase fraction were checked as follows: *p*-Nitrophenylphosphatase activity was checked by measuring the *p*-nitrophenol released (106). Phosphatase against 4'-phosphopantothenic acid was checked by measuring the pantothenic acid released (99), and that against ATP by measuring the ADP and AMP formed using paper chromatography followed by the absorption measurment at 260 nm.

Other methods. Protein was determined by the method of Lowery *et al.* (107) with egg albumin as a standard. For special purposes, the concentration was also determined spectrophotometrically from the absorbance at 280 nm. The absorbance of a solution of 1 mg/ml, determined by the method of Lowry *et al.* (107) was about 1.15 at 280 nm in a 1 cm cuvette with 0.01 M potassium phosphate buffer, pH 7.0.

RESULTS

Purification of the enzyme

All operations were performed at 0-5°C throughout the purification procedures. In these procedures, centrifugation was carried out at 12,000 x *g* for 30 min, and potassium phosphate buffer, pH 7.0, was used.

Preparation of crude extract. Frozen cell suspension (about 25 g as dry matter in 500 ml of 0.01 M buffer) was thawed, disrupted with a Kaijo-Denki 19 kHz ultrasonic oscillator for 4 hr, centrifuged, and the clear supernatant was obtained.

Ammonium sulfate fractionation. Three lots of the above supernatant were combined, then the solution was brought to 0.6 saturation by adding solid ammonium sulfate slowly and with stirring. The pH of the solution was maintained at 7.0 with 7.5 N ammonium hydroxide. After stirring for a further 30 min, the precipitate was collected by centrifugation, and dissolved in 0.01 M buffer. The solution was then dialyzed overnight (16 hr) against two changes of 5 liter volumes of the same buffer.

Chromatography on DEAE-cellulose. The dialyzed solution was applied to a column (5.5 x 40 cm) of DEAE-cellulose previously equilibrated with 0.01 M buffer. After washing with 2 liters of 0.02 M buffer, the enzyme was eluted with 0.05 M buffer at a flow rate of about 300 ml/hr. Fractions were collected, and those with an activity greater than 15 units per fraction (5 ml) were pooled and concentrated to about 1 ml within 12 hr using a collodion bag (Carl Schleicher and Schüll, Dassel). A typical elution profile for pantothenate kinase is shown in Fig. 1.

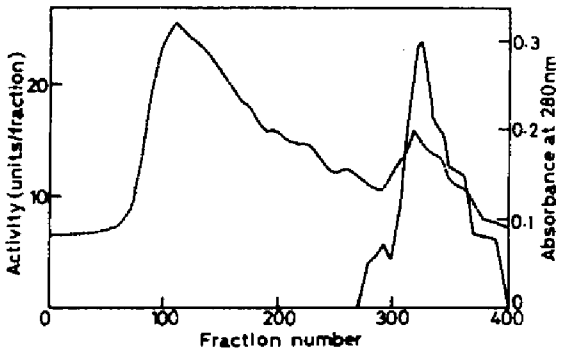

FIG. 1. Profile for the Elution of Protein and Enzyme from the Column of DEAE-cellulose.

Each fraction contains 5 ml of eluate. Details are given in the text. Solid line, enzyme activity; broken line, absorbance at 280 nm.

Chromatography on Sephadex G-150. The concentrated enzyme solution was passed through a column (1.8 x 60 cm)

of Sephadex G-150 equilibrated with 0.01 M buffer, and the enzyme was eluted with the same buffer at a flow rate of 10 ml/hr. Fractions (Nos. 22-26) with a specific activity of 31-33 were

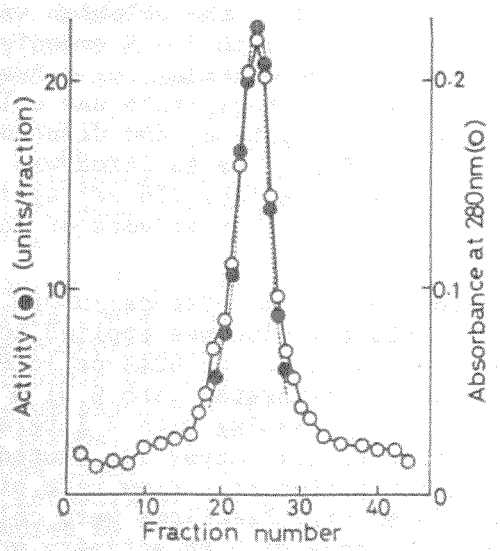

FIG. 2. Profile for the Elution of Protein and Enzyme from the Column of Sephadex G-150.

Each fraction contains 2.8 ml of eluate. Details are given in the text.

pooled, and concentrated to 1 ml using a collodion bag. A typical elution profile for the enzyme is shown in Fig. 2.

A summary of the yields and specific activities of the various fractions obtained during the purification procedure is given in Table I. The enzyme has been purified approximately 200-fold over the crude extract.

TABLE I. SUMMARY OF YIELDS AND SPECIFIC ACTIVITIES OF FRACTIONS OBTAINED DURING THE PURIFICATION OF PANTOTHENATE KINASE FROM *BREVIBACTERIUM AMMONIAGENES* IFO 12071

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
Cell extract	11305	2035	0.18
Ammonium sulfate	8750	1488	0.17
DEAE-cellulose	5.3	199.4	37.55
Sephadex G-150	2.4	82.4	34.31

Activities of phosphatases in the cell extract were negligible in degree, and they were completely separated from the kinase by the chromatography on DEAE-cellulose.

Throughout the purification procedures, rapid operations were required in order to achieve better purification. Usually, the above process was completed within 3-4 days. Stagnation in the process and further scale-up of the purification brought considerable loss of the activity. In some cases, a scale of one-third that described above was performed, and good results were obtained.

Properties of the enzyme

Polyacrylamide gel and cellulose acetate membrane electrophoresis. The purified enzyme gave a single band on disc gel electrophoresis, while the enzyme preparation whithout gel filtration on Sephadex G-150 showed one major band having the activity and three minor bands (Fig. 3). On cellulose acetate membrane,

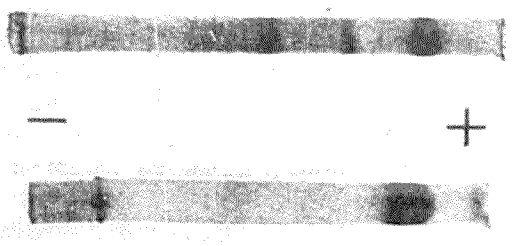

FIG. 3. Polyacrylamide Gel Electrophoresis of Pantothenate Kinase.

Top, before Sephadex G-150 gel filtration (a); bottom, after Sephadex G-150 gel filtration (b). The enzymes applied were about 100 µg (a) and 150 µg (b). Details are given in the text.

both the enzyme preparations gave a single band.

Molecular weight. The molecular weight of pantothenate kinase was determined by the method of Andrews (102) (Fig. 4). From the figure, a value of approximately 45,000 was calculated.

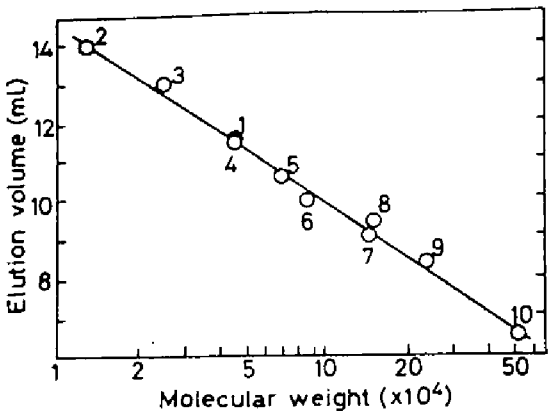

FIG. 4. Estimation of the Molecular Weight of Pantothenate Kinase by Gel Filtration.

The proteins used were: 1, pantothenate kinase; 2, cytochrome *c* (13,000); 3, chymotripsinogen A (25,000); 4, egg albumin (45,000); 5, serum albumin (67,000); 6, liver alcohol dehydrogenase (84,000); 7, aldolase (147,000); 8, yeast alcohol dehydrogenase (151,000); 9, catalase (240,000); 10, ferritin (540,000). The amount of pantothenate kinase applied was about 250 µg, and the activity was detected as described in the text. The numbered in parenthesis represents the molecular weight of each protein. Detailed conditions are givn in the text.

Absorption spectrum. The absorption spectrum of the enzyme in 0.01 M potassium phosphate buffer, pH 7.0, showed a maximum of absorbance only at 279 nm with a small shoulder at 285-290 nm. A solution of 1 mg enzyme per ml in a 1 cm cuvette exhibited an absorbance of 1.04 at 280 nm.

Stability. The purified pantothenate kinase was considerably unstable at 4°C at a protein concentration of 2 mg per ml of 0.01 M potassium phosphate buffer, pH 7.0, and lost all of its activity within 4 days. The enzyme after the DEAE-cellulose step was also inactivated completely within a week. ATP and sucrose, which stabilize the kinase of rat liver (99), were ineffective. The purified enzyme (12.2 µg in 5 µl of 0.01 M potassium phosphate buffer, pH 7.0) was incubated at various temperatures for 10 min, and the remaining activity was measured. All the intial activity remained below 30°C, but 70% of the initial activity was lost at 37°C, and at 43°C the enzyme was completely inactivated. The enzyme (12.2 µg in 50 µl of 0.01 M potassium phosphate buffer) was stable at pH 7.0 when incubated at 30°C for 10 min, but 50%, 10%, and 20% of the initial activity was lost at pH 6.5, 7.5, and 8.0, respectively.

Substrate specificity. The enzyme catalyzed not only the phosphorylation of pantothenic acid, but also that of pantothenoylcysteine, pantetheine (panthethine plus cysteine), and pantothenyl alcohol. The products of phosphorylation of pantothenic acid, pantothenoylcysteine, pantetheine, and pantothenyl alcohol were identical with 4'-phosphopantothenic acid (*Rf* 0.59 in solvent I), 4'-phosphopantothenoylcysteine (*Rf* 0.42 in solvent I), 4'-phosphopantetheine (*Rf* 0.66 in solvent I), and phosphopantothenyl alcohol* (*Rf* 0.38 in solvent I), respectively.

Nucleotide requirement. Phosphorylation of pantothenic acid by the enzyme was completely dependent on ATP and Mg^{2+}. ATP could be substituted for, partly, by ITP, GTP, and UTP. However, CTP was ineffective under the conditions employed (Table II).

TABLE II. NUCLEOTIDE SPECIFICITY OF PANTOTHENATE KINASE

The enzyme (12.2 µg; specific activity, 34.3 nmoles/mg/min) was incubated at 37°C for 15 min with 12.5 nmoles of calcium pantothenate, 0.25 µmole of $MgCl_2$, 7.5 µmoles of potassium phosphate buffer, pH 6.5, and 1.25 µmoles of nucleoside triphosphate as indicated. Details are given in the text.

Nucleotide	Relative activity
ATP	100
ITP	46
GTP	28
UTP	18
CTP	0
None	0

* This was prepared microbiologically from pantothenyl alcohol according to a similar procedure to that for the preparation of 4'-phosphopantothenic acid as described in Section VI.

Effect of divalent metal ions. The enzyme required Mg^{2+} for the activity. $MnSO_4$, $CoCl_2$, $NiCl_2$, and $ZnCl_2$ were 82%, 76%, 55%, and 52% as effective, respectively, as $MgCl_2$. $CaCl_2$, $CdCl_2$, $BaCl_2$, $PbCl_2$, $FeSO_4$, and $CuSO_4$ were ineffective. The phosphoryaltion of pantothenic acid was inhibited by the additions (10^{-3} M) of $BaCl_2$, $PbCl_2$, $FeSO_4$, and $CuSO_4$. The activities obtained with these cations were 56%, 53%, 9%, and 3% of that of the complete system ($MgCl_2$ only), respectively. $MnSO_4$, $CoCl_2$, $NiCl_2$, $ZnCl_2$, $CaCl_2$, and $CdCl_2$ showed no inhibitory effect.

Stoichiometry. When calcium pantothenate and ATP were incubated with the enzyme, 68 nmoles of pantothenic acid and 75 nmoles of ATP were consumed, and 70 nmoles of ADP were formed. The pantothenic acid was completely recovered by phosphatase digestion.

It appeared that the reaction proceeded most rapidly toward the formation of 4'-phosphopantothenic acid, since no evidence for reversibility could be obtained on incubation of small amounts of 4'-phosphopantothenic acid with ADP.

Inhibition by the intermediates of CoA biosynthesis and their related compounds. As shown in Table III, CoA and its biosynthetic precursors inhibited the phosphorylation of pantothenic acid, and CoA, especially, was a very effective inhibitor. Such inhibitions were also observed in the experiments with rat liver pantothenate kinase (98, 99). Inhibitions by these compounds seemed to be uncompetitive with pantothenic acid, according to the double reciprocal plots. The apparent *Ki* values for these compounds are also given in Table III. Pantothenoylcysteine, pantetheine, and pantothenyl alcohol, which were possible substrates for the kinase as described above, also inhibited the phosphorylation of pantothenic acid competitively. The apparent *Ki* values for pantothenoylcysteine, pantetheine, and pantothenyl alcohol were 0.31 mM, 0.20 mM, and 0.095 mM, respectively. 3',5'-ADP, ADP, 3'-AMP, AMP, GTP, GDP, ITP, UTP, CTP, NAD, NADP, and FAD showed no inhibitory effect at a concentration of 1 mM.

TABLE III. INHIBITORY EFFECT OF INTERMEDIATES OF CoA BIOSYNTHESIS ON THE PHOSPHORYLATION OF PANTOTHENATE

The enzyme (12.2 µg; specific activity, 34.3 nmoles/mg/min) was incubated for 25 min with various concentrations of intermediates as indicated. Other conditions are given in the text.

For obtaining the *Ki* values, the enzyme (8.1 µg; specific activity, 41.1 nmoles/mg/min) was incubated at 37°C for 15 min with various amounts of pantothenate, ranging from 5 to 40 nmoles, in the presence and absence of the intermediates. The amounts of the intermediates added were as follows: 12.5 nmoles of 4'-phosphopantothenic acid, 6.25 nmoles of 4'-phosphopantetheine, 6.25 nmoles of 3'-dephospho-CoA, 6.25 nmoles of CoASH, and 3.13 nmoles of CoASSCoA.

Intermediate	Inhibition (%) at 1 mM	250 µM	50 µM	*Ki* (µM)
4'-Phospho-pantothenic acid	18.8	8.8	3.8	125
4'-Phosphopanto-thenoylcysteine	-[a]	6.3	0	-
4'-Phospho-pantetheine	60.1	33.0	2.1	37
3'-Dephospho-CoA	42.5	18.8	2.5	83
CoASH	68.8	50.0	2.5	33
CoASSCoA[b]	42.5	23.8	8.8	67

a) not examined.
b) An equivalent amount to that of CoASH was added.

Kinetics. The pH optimum and the temperature optimum for the kinase reaction were found to be 6.5-7.0, and 30-37°C, respectively.

Double reciprocal plots of the reaction velocity *versus* varying concentrations of one substrate and a fixed concentration of the other substrate gave apparent *Km* values of 0.067 mM for pantothenic acid (*Vmax*, 61.6 nmoles/mg/min), 0.035 mM for ATP, and 1.0 mM for Mg^{2+}. A *Km* value for the ATP-Mg^{2+} 1:1 mixture was 1.0 mM. Substrate inhibition was observed with respect to pantothenic acid at concentrations above 0.5 mM.

Effect of inhibitors. The enzyme (12.2 µg) was not inactivated by 1 mM monoiodoacetate, 1 mM *p*-chloromercuric

benzoate, 1 mM α,α'-dipyridyl, 1 mM citrate, and 10 mM KCN, when incubated in 100 µl of 0.015 M potassium phosphate buffer, pH 6.5, at 10°C for 15 min. While the presence of 1 mM EDTA, 0.1 mM $HgCl_2$, 0.1 mM Na_2HAsO_4, and 1 mM $AlCl_3$ decreased the original activity by 83%, 100%, 77%, and 91%, respectively.

Ammonium ion also inhibited the reaction. NH_4Cl at 0.1 mM, 1 mM, and 10 mM inhibited the activity by 22%, 35%, and 50%, while $(NH_4)_2SO_4$ at 0.05 mM, 0.5 mM, and 5 mM inhibited it by 17%, 40%, and 66%, respectively.

DISCUSSION

This section describes the purification and properties of the pantothenate kinase of *Brevibacterium ammoniagenes* IFO 12071. The enzyme was purified as a homogeneous protein with a specific activity of more than 30 nmoles/mg/min from the cell extract of the bacterium. Experiments with the purified enzyme showed that the enzyme catalyzes the formation of 4'-phosphopantothenic acid and ADP from pantothenic acid and ATP in the presence of Mg^{2+}.

Cell extract of *Brevibacterium ammoniagenes* is a much richer source of pantothenate kinase than liver extracts used by Karasawa *et al.* (98) and by Abiko *et al.* (15,99). It seems probably that phosphatase activities are very week in this bacterium. Possibly this initial advantage is reflected also in gaining preparations of pantothenate kinase of much higher specific activity than those reported by Abiko *et al.* (99) for liver pantothenate kinase.

Although bacterial pantothenate kinase and liver pantothenate kinase are broadly similar, their properties differ in detail. Both the enzymes are similar in that both phosphorylate not only pantothenic acid but also pantothenoylcysteine, pantetheine, and pantothenyl alcohol, and that the reaction is inhibited by CoA and its biosynthetic intermediates. Both the enzymes are considerably unstable. The liver enzyme was relatively stable in the presence of 1 mM ATP and 10% sucrose. However, these compounds were ineffective for the bacterial enzyme. Difficulty in purifying the enzyme may be attributed to the fact that the enzyme is hard to stabilize. In the present case, with increasing purity a decrease in stability was brought about. The purified enzyme was inactivated very rapidly even at 4°C. The loss of the activity was apparently irreversible.

As previously described, incubation of pantothenic acid, cysteine, and ATP with dried cells of *Brevibacterium ammoniagenes* gave CoA in high yields. Further, cultivation of the bacterium with pantothenic acid, cysteine, AMP, and a surfactant gave a higher accumulation of CoA (2-5.5 mg/ml), and that with UMP and CMP replacing AMP gave an accumulation of 4'-phosphopantetheine, but not CoA, in the culture broth. The author initiated the present investigation in order to clear up the mechanism of this higher accumulation of CoA by the bacterium. Unexpectedly, it was observed in the bacterium as well as in rat liver, that phosphorylation of pantothenic acid, the first step in CoA biosynthesis, was strongly inhibited by CoA as the end product. Karasawa *et al.* (98) and Abiko *et al.* (99) have suggested that the inhibition of the kinase by CoA may be involved in regulating the CoA level in rat liver. The physiological significance of this inhibition may be appreciated from the standpoint of the control of CoA biosynthesis. However, the overproduction of CoA by the bacterium and this regulation mechanism in the bacterium seem to contradict each other. In this connection, further detailed investigation would be required. Another interesting property of the enzyme is its partial lack of specificity with regard to the nucleotide. Accumulation of 4'-phosphopantetheine, but not CoA, from pantothenic acid, cysteine, UMP, and CMP by the bacterium may be attributed to this broad specificity of the nucleotide for the phosphorylation of pantothenic acid (see Section VII).

Section IX.

Some Aspects of the Enzyme Activities Involved in Coenzyme A Biosynthesis in Various Microorganisms[k)]

The distribution of the enzyme activities relating to CoA biosynthesis from pantothenic acid in various microorganisms and the effect of CoA on these activities are described.

High activities of partial reactions involved in CoA biosynthesis were surveyed in various type culture strains involving bacteria, actinomycetes, lactic acid bacteria, molds, and yeasts. Generally, higher activities were found in bacteria. CoA inhibited the phosphorylation of pantothenic acid, and resulted in a decrease of CoA production in all the CoA producing strains, while only a little inhibition by CoA was observed in the other reactions, and CoA production from 4'-phosphopantothenic acid by *Brevibacterium ammoniagenes* IFO 12071 was not repressed even in the presence of 4 mM of CoA. Extracellular excretion of the enzymes of CoA biosynthesis was observed when cells were in contact with sodium laurylsulfate. Degrading activity against CoA and that against AMP were relatively lower in CoA producing strains when compared with those in other strains. It was confirmed that Brown's route of CoA biosynthesis operates in *Brevibacterium ammoniagenes* IFO 12071.

INTRODUCTION

In the previous sections, the author has described a higher production of CoA from pantothenic acid, cysteine, and AMP or ATP by *Brevibacterium ammoniagenes* IFO 12071, and has established a new process for the production of CoA. Further, including CoA itself, all the intermediates of CoA biosynthesis from pantothenic acid, though 4'-phosphopantothenoylcysteine is an exception, have been synthesized freely with high yields by the microbial process.

This section describes some aspects of the enzyme activities relating to CoA biosynthesis from the viewpoint of higher accumulation of CoA.

MATERIALS AND METHODS

Chemicals. Pantothenoylcysteine and 4'-phosphopantothenoylcysteine were the kind gifts of Dr. M. Shimizu, Daiichi Seiyaku Co., Ltd., Tokyo. All other reagents used were the same as those in the preceding section.

Microorganisms and cultivations. *Brevibacterium ammoniagenes* IFO 12071 and other strains used were strains preserved in the laboratory of Applied Microbiology, Department of Agricultural Chemistry, Kyoto University. The media for bacteria and yeasts were the same as described in Section I. Molds were grown in a medium composed of 3 g of sucrose, 0.3 g of yeast extract, 0.2 g of $NaNO_3$, 0.05 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of KCl, 0.01 g of $FeSO_4 \cdot 7H_2O$, and 100 ml of tap water, pH 6.2. Actinomycetes were grown in a medium composed of 1 g of peptone, 0.1 g of meat extract, 0.1 g of yeast extract, 0.5 g of NaCl, and 100 ml of tap water, pH 7.0. Lactic acid bacteria were grown in a medium composed of 1 g of glucose, 1 g of peptone, 1 g of yeast extract, 1 g of sodium acetate, 0.05 g of KH_2PO_4, 0.05 g of K_2HPO_4, 0.02 g of $MgSO_4 \cdot 7H_2O$, 0.001 g of $FeSO_4 \cdot 7H_2O$, 0.001 g of $MnSO_4 \cdot 4\text{-}6 H_2O$, 0.001 g of NaCl, and 100 ml of tap water, pH 7.0. All the cultivations were carried out with shaking for 2-4 days at 28°C, except for lactic acid bacteria, which were cultured without shaking.

Preparation of dried cells. Dried cells of various microorganisms were prepared according to the method described previously (see Section I).

Preparation of cell extract. Enzyme extraction was carried out as follows: (A) Washed cells of *Brevibacterium ammoniagenes* IFO 12071 (700 mg as dry matter) were suspended in 20 ml of 0.01 M potassium phosphate buffer, pH 7.0, containing 0.03% 2-mercaptoethanol, disrupted by a Kaijo-Denki 19 kHz ultrasonic oscillator for 10 min below 10°C, and centrifuged. The supernatant was dialyzed overnight against 2 liter volumes of the same buffer at 4°C, which was then diluted to a protein concentration of 6.6 mg per ml with the same buffer and used as the enzyme. (B) The washed cells (700 mg as dry matter), 14 mg of sodium laurylsulfate, and 350 μmoles of potassium phosphate buffer, pH 7.0 were incubated for 1 hr at 20°C in a total volume of 7 ml, and the cells were removed by centrifugation. The supernatant was used as the enzyme.

Reactions and activity measurements. The reaction mixture for the synthesis of CoA from pantothenic acid contained, in 0.5 ml, 1 μmole of sodium pantothenate, 2 μmoles of cysteine, 5 μmoles of ATP, 75 μmoles of potassium phosphate buffer, pH 6.5, 5 μmoles of $MgSO_4$, 0.5 mg of sodium laurylsulfate, and 50 mg of dried cells. The activity was measured by measuring CoA formed with the phosphotransacetylase method of Stadtman *et al.* (43) using the enzyme of *Escherichia coli* Crookes AKU 0001 as described previously (see Section I). The mixture, with cysteine omitted, was used for the phosphorylation of pantothenic acid. The activity was measured by following the disappearance of pantothenic acid with *Saccharomyces carlsbergensis* ATCC 9080 (46). This was checked by measuring the recovery of pantothenic acid after digestion with alkaline phosphatase (calf intestinal mucosa). The reaction mixture for the coupling reaction between 4'-phosphopantothenic acid and cysteine contained, in 0.5 ml, 1 μmole of 4'-phosphopantothenic acid, 2 μmoles of cysteine, 5 μmoles of CTP or ATP, 5 μmoles of $MgSO_4$, 75 μmoles of potassium phosphate buffer, pH 6.5, 0.5 mg of sodium laurylsulfate, and 50 mg of dried cells. The activity was measured as the difference between the amount of 4'-phosphopantothenic acid initially present and that remaining after the reaction. The reaction mixture for the synthesis of CoA from 4'-phosphopantetheine contained, in 0.5 ml, 1 μmole of 4'-phosphopantetheine, 5 μmoles of ATP, 5 μmoles of $MgSO_4$, 75 μmoles of potassium phosphate buffer, pH 6.5, 0.5 mg of sodium laurylsulfate, and 50 mg of dried cells. A mixture in which 4'-phosphopantetheine was replaced with an equimolar amount of 3'-dephospho-CoA was used for the synthesis of CoA from 3'-dephospho-CoA. Both the activities were measured by measuring the CoA formed. The assay mixture for CoA degrading activity contained, in 0.5 ml, 0.5 μmole of CoA, 5 μmoles of $MgSO_4$, 75 μmoles of potassium phosphate buffer, pH 6.5, 0.5 mg of sodium laurylsulfate, and 30 mg of dried cells, and the activity was measured by following the disappearance of CoA in the reaction mixture. The assay mixture for AMP degrading activity contained, in 0.5 ml, 5 μmoles of AMP, 5 μmoles of $MgSO_4$, 75 μmoles of potassium phosphate buffer, pH 6.5, 0.5 mg of sodium laurylsulfate, and 50 mg of dried cells. The activity was checked by measuring the AMP remaining, using paper chromatography followed by absorption measurement at 260 nm. The solvent used was isobutyric acid-0.5 N ammonium hydroxide-0.1 M EDTA (100:60:1.6, by vol.). In all the cases, the reaction was carried out at 37°C in a total volume of 0.5 ml, and terminated by immersing the tube in boiling water for 5 min. After 1.5 ml of water was added, cells were removed by centrifugation. The supernatant was employed in the determination of the substrate remaining or the product formed.

Other method. Protein was determined by the method of Lowry *et al.* (107).

RESULTS

Distribution of the enzyme activities relating to CoA biosynthesis in various microorganisms and the effect of CoA on the activities

In Section I, it has been shown that *Brevibacterium ammoniagenes* IFO 12071 and several bacteria are able to synthesize CoA from pantothenic acid, cysteine, and ATP with high yields. In the present experiment, higher activities of the partial reactions involved in CoA biosynthesis were surveyed in further varieties of microorganisms, which involved 29 strains of bacteria, 22 strains of actinomycetes, 21 strains of lactic acid bacteria, 15 strains of molds, and 31 strains of yeasts. High activities for the phosphorylation of pantothenic acid were found in 12 strains of bacteria, 2 strains of actinomycetes, and 1 strain of mold, by which more than 40% of the pantothenic acid added was phosphorylated. Wide distribution of the coupling activity between 4'-phosphopantothenic acid and cysteine was found. In 22 strains of bacteria, 6 strains of actinomycetes, 5 strains of lactic acid bacteria, and 2 strains of molds, more than 40% of the 4'-phosphopantothenic acid added coupled with cysteine in the presence of CTP. CTP was a more preferential energy source than ATP in almost all the strains tested, as pointed out by Brown (14) in *Proteus morganii*. But, *Bacillus megaterium* NI 8100 and *Bacillus subtilis* IFO 3007 showed a higher activity with ATP than with CTP. Eleven strains of bacteria, 2 strains of actinomycetes, 2 strains of lactic acid bacteria, and 1 strain of mold produced CoA from 4'-phosphopantetheine with more than 40% yield. Thirteen strains of bacteria, 3 strains of actinomycetes, 2 strains of lactic acid bacteria, and 2 strains of molds converted more than 40% of the 3'-dephospho-CoA added to CoA. Among them, 2 strains of *Brevibacterium ammoniagenes* IFO 12071 and IFO 12072, and *Corynebacterium glutamicum* ATCC 13060 gave an almost quantitative conversion of 3'-dephospho-CoA

TABLE I. ACTIVITIES OF THE ENZYMES INVOLVED IN CoA BIOSYNTHESIS IN SEVERAL MICROORGANISMS AND THE EFFECT OF CoA

All the reactions were carried out for 4 hr with or without CoA. Other conditions are described in the text. The amount of CoA supplemented was 0.5 μmole. Activities are given as μmole of the product formed or the substrate consumed in 0.5 ml of the reaction mixture. The activities with CoA in each reaction are given in the right hand column of the table.

Strain	Enzyme activity									
	PaA → CoA		PaA → P-PaA		P-PaA[a] → P-PaCySH		P-PaSH → CoA		DP-CoA → CoA	
Brevibacterium ammoniagenes IFO 12071	1.0	0.5	1.0	0.8	1.0	1.0	1.0	1.0	1.0	0.9
" " IFO 12072	0.7	0.3	0.9	0.8	1.0	1.0	0.8	0.8	1.0.	0.9
" sp. AKU 0644	0.4	0.1	0.6	0.2	0.9	1.0	0.6	0.5	1.0	0.9
Corynebacterium glutamicum ATCC 13059	0.5	0.1	0.8	0.4	0.9	0.9	0.7	0.6	0.9	0.9
" " ATCC 13032	0.4	0.1	0.8	0.4	0.7	0.8	0.7	0.5	0.8	0.8
Sarcina lutea IFO 3232	0.5	0.2	0.7	0.4	0.7	0.6	0.6	0.7	0.9	0.9
Micrococcus luteus IFO 3763	0.6	0.3	0.7	0.5	1.0	1.0	0.8	0.7	0.7	0.5
Nocardia corallina IFO 3338	0.4	0.1	0.6	0.4	0.8	0.6	0.6	0.5	1.0	0.7
Escherichia freundii K1 AKU 0011	tr[b]	tr	tr	tr	0.9	0.9	0.2	tr	0.1	tr
Flavobacterium fuscum AKU 0140	tr	tr	tr	tr	0.8	0.9	0.1	tr	0.5	0.3
Bacillus subtilis IFO 3007	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
Aspergillus niger IFO 4091	tr	tr	0.5	0.5	0.1	0.1	0.4	0.4	0.5	0.5

a) Activities were measured in the reaction mixture with CTP.
b) tr: trace.

Abbreviations used: PaA, pantothenic acid; P-PaA, 4'-phosphopantothenic acid; P-PaCySH, 4'-phosphopantothenoylcysteine; P-PaSH, 4'-phosphopantetheine; DP-CoA, 3'-dephospho-CoA.

to CoA up to a concentration of 8 mM. As to the over all reaction to produce CoA from pantothenic acid, cysteine, and ATP, higher activities were shown by 8 strains of bacteria and 1 strain of actinomycete, which accumulated CoA at a concentration of more than 0.5 mg per ml. A part of the results is shown in Table I. Table I also shows the effect of CoA on the activities of the enzymes involved in CoA biosynthesis in several microorganisms. CoA inhibited the phosphorylation of pantothenic acid, and resulted in the decrease of CoA production in all the strains tested, while no significant inhibition was observed in the formation of CoA from 4'-phosphopantetheine and from 3'-dephospho-CoA, and in the coupling between 4'-phosphopantothenic acid and cysteine. Degrading activity against CoA and that against AMP were relatively lower in CoA producing strains when compared with those in other strains, as shown in Table II.

TABLE II. CoA- AND AMP-DEGRADING ACTIVITIES IN SEVERAL MICROORGANISMS

The reaction was carried out as described in the text. Activities are given as μmoles of the substrate degraded in 0.5 ml of the reaction mixture.

Strain	Degrading activity against	
	CoA[a]	AMP[b]
Br. ammoniagenes IFO 12071	0	0.15
" " IFO 12072	0	0
" sp. AKU 0644	0	0
C. glutamicum ATCC 13059	0	0
" " ATCC 13032	0.05	0.4
S. lutea IFO 3232	0	0.5
M. luteus IFO 3763	0.05	0.85
N. corallina IFO 3338	0	1.85
E. freundii K1 AKU 0011	0.5	3.15
F. fuscum AKU 0140	0.4	4.85
B. subtilis IFO 3007	0.5	5.0
A. niger IFO 4091	0.15	1.4

a) The reaction time was 2 hr.
b) The reaction time was 3 hr.

Activities for the phosphorylation of pantothenic acid and the accumulation of CoA in the culture broth were tested, using several CoA producing strains. The strains were adapted to the fermentation medium used for CoA production by *Brevibacterium ammoniagenes* IFO 12071 by repeated single colony selections, and cultivated in the fermentation medium as described previously (see Section IV). *Brevibacterium ammoniagenes* IFO 12072 and *Brevibacterium* sp. AKU 0644 gave CoA accumulation (Table III).

TABLE III. PHOSPHORYLATION OF PANTOTHENIC ACID AND ACCUMULATION OF CoA IN THE CULTURE BROTH BY SEVERAL BACTERIA

All the cultivations were carried out as described previously (see Section IV.), except that cysteine was omitted in the case of the phosphorylation of pantothenic acid yielding 4'-phosphopantothenic acid (P-PaA). Assays of CoA and P-PaA were made for 6 day cultures. Growth was checked for 3 day cultures.

Strain	Product formed[a]		Growth[b]
	P-PaA	CcA	
Br. ammoniagenes IFO 12071	2.0	3.3	0.71
" " IFO 12072	1.4	1.0	0.64
" sp. AKU 0644	1.7	0.8	0.65
" sp. AKU 0645	1.0	0.3	0.69
C. glutamicum ATCC 13059	0.9	0.1	0.54
" " ATCC 13060	1.2	0.4	0.57
" " ATCC 13032	0.4	0.1	0.41
S. lutea IFO 3232	0.8	0.1	0.60
M. luteus IFO 3763	0.1	0	0.35

a) Values are given as mg per ml. The amount of P-PaA formed is given as pantothenic acid consumed.
b) A half ml of broth was diluted to 50 ml, and the turbidity at 610 nm was read. Growth check was made only in the case of CoA formation.

Effect of CoA concentration on the accumulation of CoA

It has been previously observed that a purified pantothenate kinase of *Brevibacterium ammoniagenes* IFO 12071, as well as that of rat liver (98,99), is strongly inhibited by CoA as the end product (see Section VIII). As shown in Fig. 1, a CoA dependent inhibition of the phosphorylation of pantothenic acid was also observed in a system with dried cells of this bacterium, while only a little inhibition was observed in the coupling between 4'-phosphopantothenic acid and cysteine and that between 4'-phosphopantetheine and ATP,

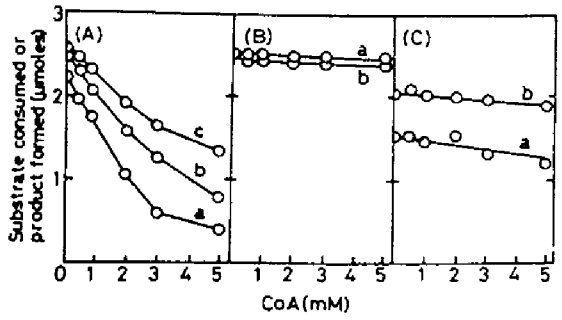

FIG. 1. Effect of CoA Concentration on the Reactions Involved in CoA Biosynthesis.

(A) Phosphorylation of pantothenic acid: The reaction was carried out for 3 hr (a), 5.5 hr (b), or 8 hr (c) with or without CoA as indicated. Other conditions were the same as those described in the text except that the pantothenic acid and ATP added were 2.5 and 7.5 μmoles, respectively.

(B) Coupling of 4'-phosphopantothenic acid with cysteine in the presence of CTP (a) or ATP (b): The reaction was carried out for 5.5 hr with or without CoA as indicated. Other conditions were the same as those described in the text except that the 4'-phosphopantothenic acid, cysteine, and CTP (or ATP) added were 2.5, 5, and 7.5 μmoles, respectively.

(C) Conversion of 4'-phosphopantetheine to CoA: The reaction was carried out for 3 hr (a) and 5.5 hr (b) with or without CoA as indicated. Other conditions were the same as those described in the text except that the 4'-phosphopantetheine and ATP added were 2.5 and 7.5 μmoles, respectively.

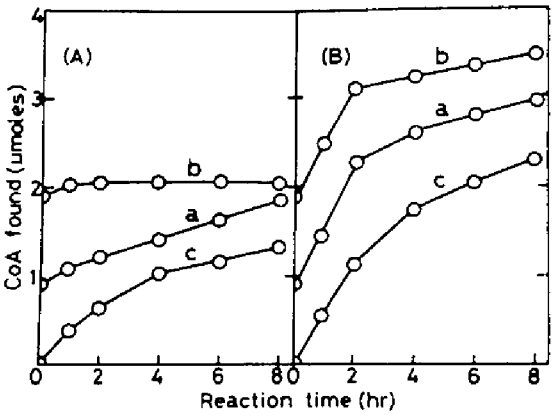

FIG. 2. Time Course for the Synthesis of CoA in the Presence of CoA.

(A) Synthesis from pantothenic acid: The reaction was carried out with 1 μmole (a) or 2 μmoles (b) of CoA. Other conditions were the same as those described in the text except that the pantothenic acid, cysteine, and ATP added were 2.5, 5, and 7.5 μmoles, respectively. The mixture without CoA was used as a control run (c).

(B) Synthesis from 4'-phosphopantothenic acid: The reaction was carried out with 1 μmole (a) or 2 μmoles (b) of CoA. Other conditions were the same as those described in the text except that the 4'-phosphopantothenic acid, cysteine, and ATP added were 2.5, 5, and 7.5 μmoles, respectively. The mixture without CoA was used as a control run (c).

even when 5 mM of CoA were present in the reaction mixture. The CoA synthesis from pantothenic acid was almost completely repressed when 4 mM of CoA were present. This inhibition could be partly overcome by increasing the concentration of the dried cells. On the other hand, the synthesis from 4'-phosphopantothenic acid was not repressed significantly, even when 4 mM of CoA were present (Fig. 2).

Stability of the enzyme activities involved in CoA biosynthesis

The enzyme activities of CoA biosynthesis in dried cells of *Brevibacterium ammoniagenes* IFO 12071 were considerably stable when stored at -15°C, and loss of the activities was within 16% after 3 years storage (Table IV). Similar results were obtained with the experiment with washed cells.

Effect of surfactant on the enzyme activities involved in CoA biosynthesis

The author has previously observed the great stimulation of CoA accumulation by the addition of certain surfactants (see Section II, III, and IV). As shown in Table V, extracellular excretion of the enzymes of CoA biosynthesis was observed, when the washed cells of *Brevibacterium ammoniagenes* IFO 12071 were treated with sodium laurylsulfate. However, the proteins which leaked out were a very small amount when compared with that in cells which were treated with ultrasonic oscillation, and substantial activity remained in the cells. The dialyzed cell extract contained activities of pantothenate kinase, 4'-phosphopantothenoylcysteine synthetase, 4'-phosphopantothenoylcysteine decarboxylase, 3'-de-

TABLE IV. EFFECT OF STORAGE TIME ON THE ENZYME ACTIVITIES OF CoA BIOSYNTHESIS IN DRIED CELLS

The reaction for the phosphorylation of pantothenic acid (PaA) was carried out for 5 hr as described in the text, except that PaA and ATP added were in the amounts of 2.5 and 7.5 μmoles, respectively. The reaction for the coupling of 4'-phosphopantothenic acid (P-PaA) with cysteine yielding 4'-phosphopantothenoylcysteine (P-PaCySH) was carried out for 3 hr as described in the text, except that the P-PaA, cysteine, CTP, and dried cells added were 2.5, 5, 7.5 μmoles, and 30 mg, respectively. The reaction for the synthesis of CoA from PaA was carried out for 5 hr as described in the text, except that the PaA, cysteine, and ATP added were 2.5, 5, and 7.5 μmoles, respectively.

Storage time (year)	Activity remaining (%)		
	PaA → P-PaA	P-PaA → P-PaCySH	PaA → CoA
0	100	-[a]	100
1	95	100	97
2	90	100	100
3	88	100	84

a) not tested.

phospho-CoA pyrophosphorylase, and 3'-dephospho-CoA kinase, which are necessary for the operation of Brown's pathway of CoA biosynthesis, but pantothenolysteine decarboxylase activity, which converts pantothenoylcysteine to pantetheine, could not be detected.

DISCUSSION

In 1954 Levintow and Novelli (12) and Hoagland and Novelli (13) proposed a pathway for the biosynthesis of CoA in animals, in which pantothenic acid couples with cysteine to yield pantothenoylcysteine as the first step, followed by decarboxylation to pantetheine, which is then phosphorylated to form 4'-phosphopantetheine, and they suggested that the pathway is as follows: pantothenic acid → pantothenoylcysteine → pantetheine → 4'-phosphopantetheine → 3'-dephospho-CoA → CoA. In 1959, However, Brown (14) proposed an alternative pathway using rat liver, rat kidney, and *Proteus morganii*, in which pantothenic acid is first phosphorylated to

TABLE V. ENZYME ACTIVITIES OF CoA BIOSYNTHESIS AND THEIR LEAKAGE FROM THE CELLS BY SURFACTANT TREATMENT

Enzyme extraction was carried out as described in the text. Twenty-five mmoles of each substrate were incubated for 30 min at 37°C with 2.5 μmoles of ATP, 0.25 μmole of $MgCl_2$, 15 μmoles of potassium phosphate buffer, pH 7.0, and 0.225 ml of the supernatant as the enzyme in a total volume of 0.25 ml, except as indicated in the footnotes. The reaction was terminated by boiling the tube for 1 min and the mixture was employed in the determination of the substrate or the product. Activities are given as mmoles of the substrate consumed or the product formed per 30 min.

Enzyme and reaction	Activity extracted by		
	ultrasonic oscillation	SLS	buffer[d]
PaA kinase (PaA → P-PaA)	15.5	4	0
P-PaCySH synthetase[a] (P-PaA → P-PaCySH)	25	14.5	4
P-PaCySH decarboxylase[b] (P-PaCySH → P-PaSH)	25	-[e]	-
DP-CoA pyrophosphorylase and DP-CoA kinase (P-PaSH → CoA)	17.5	7	0
DP-CoA kinase (DP-CoA → CoA)	19	6.5	1.5
PaCySH decarboxylase[c] (PaCySH → PaSH)	0	-	-

a) A half μmole of cysteine was supplemented and ATP was replaced by an equimolar amount of CTP.
b) ATP was omitted. The activity was checked by measuring the P-PaSH formed by *Lactobacillus bulgaricus* B1 (94) after digesting the sample with alkaline phosphatase.
c) ATP was omitted. The activity was checked by the same method as described in the footnote b).
d) The extraction conditions were the same as those with SLS except for omitting SLS.
e) not tested.

Abbreviation used: PaCySH, pantothenoylcysteine; PaSH, pantetheine; SLS, sodium laurylsulfate. Other abbreviations used here are the same as those in Table I.

yield 4'-phosphopantothenic acid prior to coupling with cysteine, and concluded that the pathway is as follows: pantothenic acid → 4'-phosphopantothenic acid → 4'-phosphopantothenoylcysteine → 4'-phosphopantetheine → 3'-dephospho-CoA → CoA. He also suggested that in

many systems it is the only operative route. Later, Abiko (15,16) and Abiko *et al.* (17) revalued these two routes in detail, and confirmed that Brown's route operates in rat liver.

The studies described in the preceding sections and the present studies showed that a CoA producing strain, *Brvibacterium ammoniagenes* IFO 12071, is able to accumulate 4'-phosphopantothenic acid and 4'-phosphopantetheine, which are the intermediates of Brown's route, and contains all the enzymes, pantothenate kinase, 4'-phosphopantothenoylcysteine synthetase, and 4'-phosphopantothenoylcysteine decarboxylase, necessary for the operation of this route. These observations suggest that Brown's route operates in this bacterium. Another result which supports this conclusion is that no pantothenoylcysteine decarboxylase activity could be detected in the bacterium. It is considered that Brown's route also operates in a wide variety of microorganisms because many of those tested contained at least one or two of the enzyme activities involved only in this route.

It was observed in many microorganisms tested, as well as in *Brevibacterium ammoniagenes* IFO 12071 (see Section VIII) and in rat liver (98,99), that phosphorylation of pantothenic acid was strongly inhibited by CoA as the end product. This suggests that a feedback inhibition of pantothenate kinase by CoA may be involved in regulating the intracellular CoA level as a general regulation mechanism. And its occurrence in wide distribution suggests that it may play a significant role in the control of CoA biosynthesis. However, the presence of this inhibition mechanism is considered an undesirble feature for obtaining CoA in a high yield. Possibly this disadvantage is reflected in the difficulty in obtaining a much higher accumulation of CoA than that the author previously attained (3-5 mg pe ml, see Section IV). Some device to avoid it may be possible: Derivation of a mutant lacking the control mechanism or development of a method for the specific removal of the product from the synthesizing system may bring a much higher accumulation of CoA. The interesting finding presented in this section is that the other enzymes involved in CoA biosynthesis were less sensitive against CoA. The higher accumulation of CoA from 4'-phosphopantothenic acid and cysteine in the presence of CoA may be attributed to this less-sensitiveness of the enzymes. This route for synthesizing CoA is probably one of the available routes for avoiding the feedback inhibition.

Another interesting finding is that all the CoA producing strains possess considerably low degrading activities against CoA and AMP. This seems to be one of the improtant features necessary for the production of CoA. Probably this may be partly reflected in the synthesizing activities of CoA as indicated in Table I.

The removal of permeability barriers of the cells was confirmed by the leakage of the enzymes of CoA biosynthesis from the cells upon the addition of a surfactant. This suggests that CoA synthesis occurs partly extracellularly.

A high stability of the dried cells seems to give an advantage for its practical use as an enzyme source for the production of CoA.

CHAPTER II.
DEGRADATION OF PANTOTHENYL ALCOHOL BY MICROORGANISMS[1)]

Several microorganisms isolated from soil were found to grow in the medium containing pantothenyl alcohol. The results of the investigation of the degradative metabolism of this compound demonstrated that there are two different inducible pathways.

The experiments with strain 1041 showed that 3-aminopropanol appeared in the early stage of culture, and β-alanine in the stationary phase of growth without accumulation of pantothenic acid. 3-Aminopropanol plus pantoate, as well as pantothenyl alcohol, supported the growth of the induced culture. Rapid hydrolysis of pantothenyl alcohol was produced by incubating with either washed cells or cell extract of this bacterium grown on pantothenyl alcohol, while no oxidation of this substrate to pantothenic acid was observed. When 3-aminopropanol was incubated with the washed cells or cell extract, only β-alanine was a major product. Isolation and identification of the products were performed. These results led to the conclusion that pantothenyl alcohol is hydrolyzed to pantoic acid and 3-aminopropanol as the first step, which is then followed by oxidation to β-alanine.

Strain 1091 produced pantothenic acid, but not 3-aminopropanol, from pantothenyl alcohol. β-Alanine plus pantoate, as well as pantothenyl alcohol, supported the growth of the induced culture, while 3-aminopropanol plus pantoate did not support it. No degradation of 3-aminopropanol was observed. Isolation and identification of pantothenic acid and a 3-methyl-2-benzothiazolone hydrazone derivative of the aldehyde from pantothenyl alcohol were performed. From the results, it was confirmed that pantothenyl alcohol is first oxidized to pantothenic acid, which is then hydrolyzed to β-alanine and pantoic acid.

Pantothenyl alcohol was also oxidized to pantothenic acid by *Bacillus roseus* AKU 0208. The enzyme was not induced in the presence of pantothenyl alcohol.

INTRODUCTION

It has been reported that pantothenyl alcohol, an alcohol analog corresponding to pantothenic acid, is a growth inhibitor of some pantothenic acid-requiring bacteria (39,40), although it has a pantothenic acid-like activity in mammals (36-38,108-111). Abiko *et al.* (110) investigated the metabolism of this compound using rats and demonstrated its oxidative conversion to pantothenic acid by liver alcohol dehydrogenase. However, little is known of the metabolism of this alcohol in microorganisms. It seems, therefore, to be interesting to know the microbial response of this antimetabolite. This chapter describes some aspects of the degradation of pantothenyl alcohol by microorganisms.

MATERIALS AND METHODS

Chemicals. Potassium D-pantoate was prepared according to the method of Stansly and Schlosser (112). Potassium pantothenate was prepared from the calcium salt according to the procedure of

Goodhue and Snell (113). Other chemicals were from commercial sources.

Media. The basal medium was composed of 0.05 g of K_2HPO_4, 0.05 g of $MgSO_4 \cdot 7H_2O$, 1 ml of vitamin solution containing, per liter, 1 mg of thiamine·HCl, 1 mg of riboflavin, 1 mg of nicotinic acid, 0.2 mg of *p*-aminobenzoic acid, 0.01 mg of folic acid, and 0.01 mg of biotin, and 99 ml of water. It was adjusted to pH 6.8-7.0 with KOH and sterilized by autoclaving at 120°C for 10 min. The pantothenyl alcohol medium was prepared by adding a concentrated, filter-sterilized solution of D-pantothenyl alcohol to the sterilized basal medium to supply 0.5 g per 100 ml. Pantothenyl alcohol-agar was prepared by solidifying pantothenyl alcohol medium with 2% agar. The medium used for the screening of the strains accumulating pantothenic acid from pantothenyl alcohol was composed of 0.5 g of peptone, 0.5 g of meat extract, 0.05 g of yeast extract, 0.2 g of NaCl, and 100 ml of water. For yeasts the medium supplemented with 2 g of glucose was also used. The pH of the medium was adjusted to 6.8-7.0 for bacteria and actinomycetes, and to 5.5-6.0 for yeasts and molds. After autoclaving at 120°C for 15 min, filter-sterilized D-pantothenyl alcohol was added to supply 0.02 g per 100 ml.

Isolation of pantothenyl alcohol degrading strains. After the enrichment cultures on pantothenyl alcohol medium for three times, the cultures were streaked on pantothenyl alcohol-agar plates. Colonies of distinctly different appearance were picked into the liquid pantothenyl alcohol medium. These plating and shaking processes were each repeated 3 times, and 14 apparently different strains were obtained. Stock cultures were maintained by culturing for 4 days at 28°C on pantothenyl alcohol-agar stants, then storing at 5°C for 1.5 months. In the studies described herein strains 1041 and 1091 were mainly used.

Culture procedures. For small scale experiments, cultures in 5 ml of liquid medium contained in 16.5 x 160 mm test tubes were grown from 2% (by vol.) inoculum at 28°C on a reciprocal shaker (Iwashiya, RTR-1), which was set for 240 rpm with a tube angle of 20°. Larger volumes (100 ml or 500 ml) were grown in 0.5 liter or 2 liter volume of shaking flasks on a reciprocal shaker (Iwashiya, RLR-5), which was set for 120 oscillations per min with an amplitude of 7 cm. Growth was measured turbidimetrically at 610 nm, and cell yield was determined by means of a culture relating absorbance to dry cell weight.

Screening for pantothenic acid accumulating strains. Type culture strains preserved in the Laboratory of Applied Microbiology, Department of Agricultural Chemistry, Kyoto University, and the isolates utilizing pantothenyl alcohol were grown in the medium described above for 2 days at 28°C on a reciprocal shaker. After boiling the tube, the pantothenic acid in the broth was assayed.

Preparation of washed cell suspensions. Usually the cells of the exponential phase of growth were harvested by centrifugation, washed twice with 0.85% NaCl, suspended in 0.01 M potassium phosphate buffer, pH 7.0, and stored at 0°C until used.

Preparation of cell extracts. Twenty-five ml of each cell suspension, containing approximately 300 mg of cells (dry weight) per ml, were disrupted with a Kaijo-Denki 19 kHz ultrasonic oscillator for 10 min below 10°C and clarified by centrifugation at 12,000 x *g* for 15 min at 0°C, and the supernatant (protein concentration, 10-30 mg/ml) was dialyzed overnight against two changes of 2 liter volumes of 0.01 M potassium phosphate buffer, pH 7.0.

Chromatographies and electrophoresis. Paper chromatography (PPC) was carried out by the ascending technique on Toyo Roshi No.53 paper. The solvent systems used were: I, *n*-butanol-acetic acid-water (5:2:3, by vol.); II, isobutyric acid-0.5 N ammonium hydroxide (5:3, by vol.); and III, *n*-propanol-28% ammonium

hydroxide-water (6:3:1, by vol.). Thin layer chromatography (TLC) on Eastman Chromagram Sheet 6065 was carried out using the following solvents: I, *n*-butanol-acetic acid-water (5:2:3, by vol.); II, 99% ethanol-water (6:4, by vol.); and III, methanol-*n*-butanol (3:1, by vol.). Paper electrophoresis (PEP) was carried out on Toyo Roshi No.53 paper impregnated with 0.02 M acetate buffer, pH 3.5 at 0.5 mA/3 cm (500 volts) for 60 min. 3-Aminopropanol, β-alanine, and other amino acids were detected by ninhydrin (0.25% in acetone) spray. β-Alanine was also detected by bioautographic techniques using *Saccharomyces carlsbergensis* ATCC 9080 (46). Pantoic acid and other nonvolatile acids were detected by sugar-aniline reagent (114) in the earlier experiments. Pantothenyl alcohol was detected by bioautographic techniques using *Leuconostoc mesenteroides* P-60 ATCC 8042 (115,116), and pantothenic acid with *Saccharomyces carlsbergensis* ATCC 9080 (46) or *Lactobacillus plantarum* ATCC 8014 (45).

Determination of pantothenyl alcohol. Pantothenyl alcohol was separated by the electrophoretic method described above, eluted from the paper, and determined microbiologically with *Leuconostoc mesenteroides* P-60 ATCC 8042 (115, 116).

Determination of 3-aminopropanol and β-alanine. 3-Aminopropanol or β-alanine was separated by PPC with solvent I. The corresponding ninhydrin-reactive zones were cut out, and the color was extracted with methanol and estimated by reading the absorption at 600 nm against similarly treated standards of 3-aminopropanol or β-alanine. β-Alanine was also determined microbiologically with *Saccharomyces carlsbergensis* ATCC 9080 (46) after separating by PPC and eluting from the paper.

Assay of pantothenyl alcohol cleaving activity. The cleaving activity was determined routinely by measuring the disappearance of pantothenyl alcohol in the reaction mixture. This was checked by measuring the 3-aminopropanol formed. The reaction mixture contained 0.5 μmole of pantothenyl alcohol, 25 μmoles of potassium phosphate buffer, pH 7.0, and the appropriately diluted enzyme in a total volume of 0.5 ml. The mixture was incubated at 37°C for 20-60 min and the reaction was terminated by boiling the tube for 1 min.

Assay of pantothenyl alcohol oxidizing activity. The oxidizing activity was determined routinely by measuring the pantothenic acid formed. The reaction conditions were the same as those for the assay of pantothenyl alcohol cleaving activity. For special purposes, the aldehyde (pantothenyl aldehyde?) formed was measured by the 3-methyl-2-benzothiazolone hydrazone (MBTH) method of Sawicki *et al.* (117).

Assay of 3-aminopropanol oxidizing activity. The activity was determined routinely by measuring the β-alanine formed. The reaction system used was the same as described above except that pantothenyl alcohol was replaced with an equimolar amount of 3-aminopropanol.

Assay of pantothenic acid cleaving activity. The activity was determined by measuring the disappearance of pantothenic acid in the reaction mixture which initially contained 0.5 μmole of potassium pantothenate. Other conditions were the same as those for the assay of pantothenyl alcohol cleaving activity.

Other methods. Pantothenic acid was determined microbiologically by *Lactobacillus plantarum* ATCC 8014 (45), for which pantoic acid, pantolactone, and β-alanine are inactive. Pantothenyl alcohol did not inhibit for the growth of this organism under the conditions employed here. Pantoic acid was determined according to the method of Nurmikko *et al.* (118).. Protein was measured by the method of Lowry *et al.* (107). Sulfur contents of the MBTH derivatives were determined by the Schöninger combustion method (119). NMR spectra were measured on a Varian A-60 spectrometer at 60 MHz or a Hitachi Perkin-Elmer R-22 spectrometer at 90 MHz in D_2O with DSS as an internal standard.

RESULTS

Studies with isolated strains

Characterization of cultures. Culture 1041 was Gram-negative rod (0.5-0.8 x 2-3 μ), oxidative in Hugh and Leifson's medium (120), and Kovacs' oxidase (121) positive, and produced diffusible fluorescent pigments. On pantothenyl alcohol-agar it formed yellow, glistening, and smooth colonies. Culture 1091 was also Gram-negative rod (0.5-0.8 x 1-2 μ), oxidative in Hugh and Leifson's medium (120), and Kovacs' oxidase (121) positive. It produced diffusible red-brown pigments on King's A medium (122), but no fluorescence on King's B medium (122). On pantothenyl alcohol-agar it formed cream-colored and glistening colonies. Both the strains are presumably placed in the genus *Pseudomonas*.

Growth response of the isolated strains to pantothenyl alcohol and its related compounds. The comparative growth rates of strain 1041 and those of strain 1091 on pantothenyl alcohol and its hydrolyzed and oxidized products are shown in Fig. 1. In both strains, growth with pantothenate was slightly superior to that with equimolar amounts of pantothenyl alcohol itself. Both grew well with pantoate plus β-alanine, β-alanine, or pantoate plus NH_4Cl, as carbon and nitrogen source. Strain 1041 also grew with pantoate plus 3-aminopropanol or with 3-aminopropanol alone, but these were not utilized by strain 1091 during the time studied here (144 hr). The growth patterns of other strains isolated were similar to that of strain 1091.

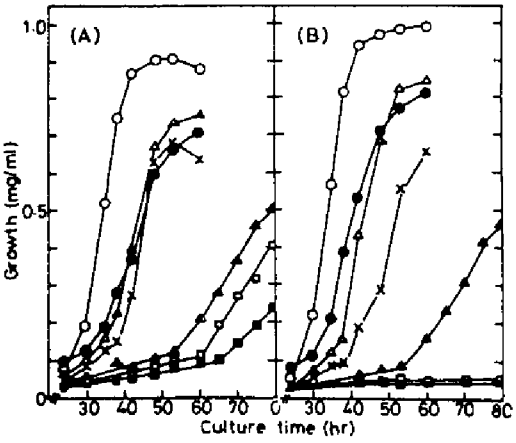

FIG. 1. Growth Response of the Isolated Strains on Pantothenyl Alcohol and its Related Compounds.

Strain 1041 (A) and strain 1091 (B) were cultivated in 500 ml shaking flasks containing 100 ml of each medium as indicated below. Growth curves were obtained in the basal medium plus: ●, pantothenyl alcohol (25 mM); ○, potassium pantothenate (25 mM); △, potassium pantoate (25 mM) plus β-alanine (25 mM); ▲, potassium pantoate (25 mM) plus NH_4Cl (25 mM); X, β-alanine (25 mM); □, potassium pantoate (25 mM) plus 3-aminopropanol (25 mM); ■, 3-aminopropanol (25 mM). All cultures were inoculated with 4 mg (dry weight) of washed cells grown in pantothenyl alcohol medium. Other conditions are given in the text.

Metabolic products formed from pantothenyl alcohol by strain 1041. Fifty μl of the filtrate from a culture of strain 1041 grown on pantothenyl alcohol medium were spotted on Toyo Roshi No.53 paper, which was then developed with either solvent I or II. After drying the papers, the products were detected by the methods described in the text. A prominent ninhydrin-reactive zone corresponding in position to 3-aminopropanol appeared when the filtrate from 35 hr culture was applied. When the filtrate from 50 hr culture was applied, however, two prominent ninhydrin-reactive zones appeared. One of them, corresponding in position to β-alanine, was active in enhancing the growth of *Saccharomyces carlsbergensis* ATCC 9080 (*Rf* 0.50 and 0.65, respectively, in solvents I and II), and the other, corresponding in position to 3-aminopropanol, was inactive (*Rf* 0.61 and 0.77, respectively, in solvents I and II). However, no detectable amounts of pantothenic acid were found.

The cells harvested from pantothenyl alcohol medium during the early logarithmic phase of growth(40 hr) rapidly cleaved pantothenyl alcohol to yield pantoic acid and 3-aminopropanol. 3-Aminopropanol was isolated through the following procedures: A mixture, containing 1.2 mmoles of pantothenyl alcohol, 2 mmoles of potassium phosphate buffer, pH 7.0, and 200 mg (dry weight) of washed cells from the early logarithmic

phase of growth (40 hr) in a total volume of 40 ml, was shaken for 18 hr at 28°C, boiled for 3 min, and centrifuged. The supernatant was applied to a column of Dowex 50W x 8 (H^+ form, 1.8 x 20 cm). After washing the column with 0.01 M HCl, 3-aminopropanol was eluted with 0.15 M HCl. Appropriate fractions were collected, treated with Dowex 1 x 2 (OH^-), adjusted to pH 3.0 with HCl, concentrated to small volume, and lyophilized (yield, 35 mg; PPC, *Rf* 0.60, 0.79, and 0.73, respectively, in solvents I, II, and III; TLC, *Rf* 0.63 and 0.78, respectively, in solvents I and II; PEP, migration towards the cathode, 11 cm; NMR at 90 MHz, (δ ppm): 1.63 (2H, p, J=3.5 Hz), 2.64 (2H, t), 3.61 (2H, t)). Formation of pantoic acid was demonstrated by the ferric hydroxamate spray (118) (PPC, *Rf* 0.52 in solvent I). Pantothenate, as well as pantothenyl alcohol, was also cleaved by the same cells. Cells of the same organism, grown with either 3-aminopropanol plus pantoate, or with pantothenate, did not cleave pantothenyl alcohol. The cells from glucose plus NH_4Cl medium were also inactive in cleaving this substrate. When 3-aminopropanol was incubated with the cells grown for 50 hr with pantothenyl alcohol, β-alanine was produced. The product was isolated through the following procedures: A mixture, containing 1.2 mmoles of 3-aminopropanol, 2 mmoles of potassium phosphate buffer, pH 7.0, and 200 mg (dry weight) of the washed cells in a total volume of 40 ml, was shaken for 22 hr at 28°C, boiled for 3 min, and centrifuged. The supernatant was passed through a column of Dowex 50W x 8 (H^+ form, 1.6 x 20 cm). After washing the column with 0.01 M HCl, the passings and washings were combined and passed through a column of Dowex 1 x 2 (chloride form, 1.6 x 20 cm), which was then washed with 0.01 M HCl. Appropriate fractions containing β-alanine were pooled and concentrated, and β-alanine was crystallized from ethanol (yield, 38 mg; PPC, *Rf* 0.53, 0.65, and 0.52, respectively, in solvents I, II, and III; TLC, *Rf* 0.63 and 0.62, respectively, in solvents I and II; PEP, migration towards cathode, -0.5 cm; NMR at 90 MHz (δ ppm): 2.54 (2H, t), 3.18 (2H, t)).

When dialyzed crude extract from the cells grown with pantothenyl alcohol was incubated with this substrate, a clear ninhydrin-reactive zone of 3-aminopropanol appeared on chromatograms in addition to a faint ninhydrin-reactive zone of β-alanine. Similarly, pantoic acid was readily detected by the ferric hydroxamate spray (118). The extract from the cells of the early logarithmic growth phase (35 hr) had the highest activity of pantothenyl alcohol cleavage (38 nmoles pantothenyl alcohol decomposed per mg per hr). The activities of the cells grown for 45 hr, 55 hr, and 70 hr were 73%, 58%, and 27%, respectively, of that of the cells grown for 35 hr. Pantothenic acid was not produced from pantothenyl alcohol, though it was cleaved to form β-alanine and

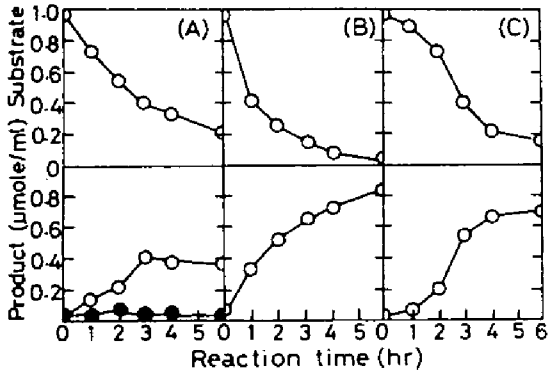

FIG. 2. Reactions Involved in the Degradation of Pantothenyl Alcohol by Strain 1041.

(A) Degradation of pantothenyl alcohol: The reaction was carried out with 4.15 mg of the crude enzyme. Other conditions are given in the text. Top, remaining pantothenyl alcohol; bottom, ●, pantothenic acid found; ○, 3-aminopropanol found.

(B) Oxidation of 3-aminopropanol to β-alanine: The reaction was carried out with 4.15 mg of the crude enzyme. Other conditions are given in the text. Top, remaining 3-aminopropanol; bottom, β-alanine found.

(C) Degradation of pantothenic acid: The reaction was carried out with 1.25 mg of the crude enzyme. Other conditions are given in the text. Top, remaining pantothenic acid; bottom, β-alanine found.

The crude enzyme was prepared from the cells grown with pantothenyl alcohol for 40 hr according to the method as described in the text.

pantoic acid. 3-Aminopropanol production from DL-pantothenyl alcohol was about 50% of that from D-pantothenyl alcohol under conditions in which the cleavage of D-pantothenyl alcohol was complete. 3-Aminopropanol was oxidized to β-alanine; for the oxidation, neither NAD nor NADP was required in either the dialyzed crude extract or the precipitates with ammonium sulfate (0.25-0.50 saturation). Part of the results obtained from the experiments with crude extract is shown in Fig. 2.

Metabolic products formed from pantotenyl alcohol by strain 1091. Strain 1091 accumulated pantothenic acid in the culture broth when grown in pantothenyl alcohol medium. The accumulation occurred after the first 30 hr and increased linearly with increasing the cell growth. The pantothenic acid formed disappeared rapidly during the stationary phase of growth. The maximum accumulation was 1.5 μmoles/ml, obtained from 44 hr culture. A ninhydrin-reactive zone corresponding in position to β-alanine, but not 3-aminopropanol, appeared on paper chromatograms, when the filtrate from 50 hr culture was applied (*Rf* 0.65 and 0.53, respectively, in solvents II and III). This was active in enhancing the growth of *Saccharomyces carlsbergensis* ATCC 9080.

The washed cells grown with pantothenyl alcohol for 40 hr rapidly oxidized this substrate to pantothenic acid, which was isolated as follows: A mixture, containing 0.8 mmole of pantothenyl alcohol, 2 mmoles of potassium phosphate buffer, pH 7.0, and 200 mg (dry weight) of washed cells from the early logarithmic growth phase (35 hr) in a total volume of 40 ml, was shaken for 15 hr at 28°C, boiled for 3 min, and centrifuged. The supernatant was applied to a column of Dowex 1 x 2 (chloride form, 1.6 x 20 cm). After washing the column with water, pantothenic acid was eluted with 0.007 M HCl. Appropriate fractions were collected, neutralized with $Ca(OH)_2$, and concentrated to dryness. The residue was dissolved in a small volume of water and insoluble materials were centrifuged off. The supernatant was evaporated and the residue was again dissolved in a small volume of water. These operations were repeated until a clear solution was obtained. This solution was evaporated to reduced volume, and the calcium salt of pantothenic acid was precipitated by adding ether (yield, 103 mg; PPC, *Rf* 0.85, 0.74, and 0.80, respectively, in solvents I, II, and III; NMR at 60 MHz (δ ppm): 0.90 (3H, s), 0.93 (3H, s), 2.42 (2H, t, J=7 Hz), 3.44 (2H, t, J=7 Hz), 3.44 (2H, t), 3.98 (1H, s)). In order to detect the formation of the aldehyde from pantothenyl alcohol, the supernatant from a similar reaction mixture (40 ml) was passed through columns of Dowex 1 x 2 (OH^-, 18 ml) and then Dowex 50W x 8 (H^+, 20 ml). The passings and washings were concentrated to small volume, part of which was applied to TLC. A single blue zone was obtained after treating with MBTH spray reagent (117) (*Rf* 0.83 in solvent I). No blue zone was detected when pantothenyl alcohol or pantothenate was applied. The MBTH derivative of the aldehyde was isolated from the above concentrated solution as follows: The solution was combined with 5 ml of 0.1 M of free MBTH solution, left for 3 hr, and concentrated to dryness. The residue was dissolved in a small volume of ethanol, insoluble materials were filtered off, and the filtrate was concentrated to dryness. The residue was dissolved in a small volume of water, and the azine crystallized was collected, and recrystallized twice from water (yield, 28 mg; UV λmax in 10% ethanol, 314 nm; PPC, *Rf* 0.38 in solvent I; TLC, *Rf* 0.73 in solvent III). Hydrolysis with 0.1 M KOH and with 0.1 M HCl gave pantoic acid and pantolactone, respectively. Color yield of the MBTH derivative after oxidation with $FeCl_3$ (Sawicki reaction (117)) was observed (λmax: 618, 668 nm). Neither pantothenyl alcohol plus MBTH, nor pantothenate plus MBTH yielded the color. The same cells also cleaved pantothenate to yield β-alanine and pantoic acid, which were identified by use of similar methods to those described in the experiments with strain 1041. Cells of

the same organism grown either with pantothenate or with glucose did not oxidize pantothenyl alcohol to pantothenic acid.

Dialyzed crude extract from the cells grown with pantothenyl alcohol oxidized this substrate to pantothenic acid. But oxidation of 3-aminopropanol to β-alanine was not catalyzed by the same extract under the conditions employed. The extract from the cells from the early logarithmic growth phase (35 hr) had the highest activity (65 nmoles pantothenyl alcohol oxidized per mg per hr) of the oxidation, and the activity gradually decreased with growth of the cells. The activities with the cells grown for 45 hr, 55 hr, 70 hr, and 100 hr were 64%, 42%, 30%, and 13%, respectively, of that with the cells grown for 35 hr. Neither NAD nor NADP was required for the oxidation. When the

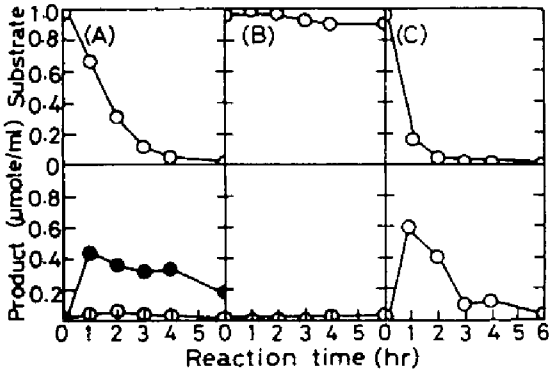

FIG. 3. Reactions Involved in the Degradation of Pantothenyl Alcohol by Strain 1091.

(A) Oxidation of pantothenyl alcohol to pantothenic acid: The reaction was carried out with 3.05 mg of the crude enzyme. Other conditions are given in the text. Top, remaining pantothenyl alcohol; bottom, ●, pantothenic acid found; ○, 3-aminopropanol found.

(B) Degradation of 3-aminopropanol: The reaction was carried out with 4.55 mg of the crude enzyme. Other conditions are given in the text. Top, remaining 3-aminopropanol; bottom, β-alanine found.

(C) Degradation of pantothenic acid: The reaction was carried out with 1.05 mg of the crude enzyme. Other conditions are given in the text. Top, remaining pantothenic acid; bottom, β-alanine found.

The crude enzyme was prepared from the cells grown with pantothenyl alcohol for 40 hr according to the method as described in the text.

aldehyde formation was assayed by Sawicki reaction (117), the increase in absorbance at 570 nm with elapse of reaction time was observed. No increase in absorbance was observed in either of the mixtures without pantothenyl alcohol or with pantothenate substituted for pantothenyl alcohol. The same extract contained a pantothenate-hydrolyzing activity, and β-alanine formation from DL-pantothenate was about 50% of that from D-pantothenate under conditions giving complete cleavage of D-pantothenate. Part of the results obtained from the experiments with crude extract is shown in Fig. 3.

Conversion of pantothenyl alcohol to pantothenic acid by Bacillus roseus AKU 0208 and other strains

Activities of the accumulation of pantothenic acid from pantothenyl alcohol were surveyed in various microorganisms; 138 strains of bacteria, 205 strains of actinomycetes, 111 strains of molds, and 195 strains of yeasts. Among them *Bacillus roseus* AKU 0208 and *Serratia plymuthicum* AKU 0062 quantitatively converted it to pantothenic acid. *Nocardia corallina* IFO 3338, *Streptomyces* sp. AKU 2505, and *Sarcina lutea* IFO 3232 also showed high accumulation of pantothenic acid. However, strain 1091 and other pantothenyl alcohol utilizing strains showed relatively lower accumulating activity under the conditions tested. In the following experiments, *Bacillus roseus* AKU 0208 was used.

Bacillus roseus AKU 0208 did not grow on pantothenyl alcohol medium. Neither pantothenate nor its hydrolyzed products was utilized as carbon and nitrogen source. The whole broth and the washed cells rapidly converted pantothenyl alcohol to pantothenic acid. The converting activity in the cell was independent of pantothenyl alcohol supplement to the medium. No degradation of pantothenate was observed though contact with the washed cells or cell extract for 6 hr at 28°C. Isolation of pantothenate was carried out as follows: A mixture, containing 3 mmoles of

pantothenyl alcohol, 1.26 mmoles of potassium phosphate buffer, pH 7.0, and 258 mg (dry weight) of washed cells in a total volume of 60 ml, was shaken for 48 hr at 28°C. The supernatant from the reaction mixture was treated as described above to give the calcium salt (yield, 466 mg; PPC, *Rf* 0.84, 0.74, and 0.79, respectively, in solvents I, II, and III; NMR at 60 MHz (δ ppm), 0.90 (3H, s), 0.93 (3H, s), 2.42 (2H, t, *J*=7 Hz), 3.44 (2H, t, *J*=7 Hz), 3.44 (2H, t), 3.98 (1H, s)). For the isolation of the aldehyde from pantothenyl alcohol, the same mixture, supplemented with 1.3 mmoles of free MBTH, was shaken for 40 hr at 28°C. After removing the cells by centrifugation, the supernatant was boiled for 1 min, cooled, concentrated to small volume, and left at 5°C overnight. The crystalline MBTH derivative of the aldehyde formed was collected and recrystallized twice from water (yield, 236 mg; UV λmax in 10% ethanol, 314 nm; PPC, *Rf* 0.35 in solvent I; TLC, *Rf* 0.70 in solvent III; sulfur content, 8.76% (required, 8.80%)).

DISCUSSION

Pantothenyl alcohol, an alcohol analog of pantothenic acid was reported to inhibit the growth of pantothenic acid-requiring bacteria (39,40), but several strains isolated from soil were found to be capable of growing in the medium containing it as a sole carbon and nitrogen source as described here. The results of the investigation on the degradative metabolism of this compound in these strains demonstrated that there are two different inducible pathways.

The experiments with strain 1041 showed that 3-aminopropanol appeared in the early stage of the culture, and β-alanine in the stationary phase of growth without accumulation of pantothenic acid. These, together with the observation that 3-aminopropanol plus pantoate, as well as pantothenyl alcohol, supported the growth of the induced culture suggest that pantothenyl alcohol is hydrolyzed to yield 3-aminopropanol and pantoic acid as the first step. Another result supporting this conclusion is that rapid hydrolysis of pantothenyl alcohol occurred during incubation with either washed cell suspension or cell extract of the bacterium grown on pantothenyl alcohol, while no oxidation of this substrate to pantothenic acid was observed. When 3-aminopropanol was incubated with the washed cells or cell extract, only β-alanine was detected as a major product. This observation suggests that the oxidation of 3-aminopropanol to β-alanine is nessesary for the further metabolism of 3-aminopropanol.

On the other hand, the degradative pathway operating in strain 1091 seems to be different from that operating in strain 1041. In the filtrate from cultures of strain 1091 grown in pantothenyl alcohol medium no 3-aminopropanol appeared, while both pantothenic acid and β-alanine were easily detected. Pantothenate and its hydrolytic products, *i.e.* β-alanine or β-alanine plus pantoate, as well as pantothenyl alcohol, supported the induced culture of the bacterium. However, 3-aminopropanol or 3-aminopropanol plus pantoate, the hydrolytic products of pantothenyl alcohol, did not support the growth of the same organism. The cell extract and the washed cell suspension rapidly oxidized pantothenyl alcohol to pantothenic acid. These observations strongly suggest that pantothenyl alcohol is first oxidized to pantothenic acid, which is then hydrolyzed to β-alanine and pantoic acid. Isolation of the aldehyde as the intermediate in the oxidation of pantothenyl alcohol to pantothenic acid may give another piece of evidence for this conclusion.

The further metabolism of pantoic acid and β-alanine formed from pantothenyl alcohol in these organisms has not been investigated, since the metabolic fate of these compounds in other organisms is rather well known (123-130).

Puisto and Nurmikko (131) and Mantsala and Nurmikko (132) reported that *Pseudomonas fluorescens* P-2 contains an inducible amidase, pantothenate hydrolase, which hydrolyzes pantothenate to pantoic acid and β-alanine. The enzyme

was induced in the presence of pantothenate. But pantothenyl alcohol was ineffective either as an inducer or as a substrate for the enzyme. Conversely, as shown in Table I, cell extract of

TABLE I. HYDROLASE ACTIVITIES AGAINST PANTOTHENYL ALCOHOL AND PANTOTHENIC ACID IN STRAIN 1041

The cultivation was carried out as described in Fig. 1 except for carbon and nitrogen sources, and cells of logarithmic phase of growth were harvested. The method for the preparation of the crude extract is described in the text. Activities are given as nmoles/mg/hr.

Carbon and nitrogen source	Hydrolase activity against	
	pantothenyl alcohol	pantothenic acid
Pantothenyl alcohol (25 mM)	32	73
Pantothenic acid (25 mM)	0	97
Glucose (25 mM) + NH_4Cl (25 mM)	0	15

strain 1041 grown with pantothenyl alcohol hydrolyzed not only this compound but also pantothenate, while that grown with pantothenate did not attack pantothenyl alcohol. Probably pantothenyl alcohol hydrolase and pantothenate hydrolase are different from each other. It is uncertain, however, whether the former can also attack pantothenate, since both enzymes were separated from each other in the present experiment.

Pantothenyl alcohol was also oxidized to pantothenic acid by *Bacillus roseus* AKU 0208. This organism did not grow on either pantothenyl alcohol or pantothenate. The enzyme was not induced in the presence of pantothenyl alcohol. In the preliminary experiments, it was observed that this enzyme acts on several primary alcohols including methanol, ethanol, and so on. Details will appear eleswhere.

CONCLUSION

Metabolic mechanism of microbial conversion of pantothenic acid to CoA has been investigated. Resting on the basis of the results, a new process for the production of CoA and its biosynthetic intermediates has been established. Presence of two different inducible routes for the degradation of pantothenyl alcohol has also been shown.

The activity of CoA accumulation was searched in various microorganisms. The result of screening showed that several yeasts and bacteria accumulate CoA in high yields from pantothenic acid, cysteine, and AMP or ATP. Among them, *Brevibacterium ammoniagenes* IFO 12071 was found to be the most excellent CoA producer. The yield was about 1 mg/ml. Baker's yeast also accumulated CoA (200 μg/ml) from pantothenic acid and cysteine under the condition coupled with ATP-generating system of the yeast.

Using dried cells of *Brevibacterium ammoniagenes* IFO 12071, the reaction conditions for CoA accumulation were estimated. The cells grown with acetic acid had the highest activity. Addition of surfactants to the reaction mixture brought a great acceleration of CoA accumulation. Under the suitable condition, the amount of CoA accumulated reached 2 mg/ml. CoA was obtained with a high yield from the reaction mixture. 3'-Dephospho-CoA and 4'-phosphopantothenic acid were also isolated as by-products. Further, including CoA itself, all the intermediates of CoA biosynthesis, though 4'-phosphopantothenoylcysteine is an exception, were synthesized with high yields: When cysteine was omitted from the reaction mixture, only 4'-phosphopantothenic acid was accumulated. 4'-Phosphopantothenic acid coupled with cysteine in the presence of CTP, and yielded 4'-phosphopantetheine. The direct and exclusive accumulation of 4'-phosphopantetheine, but not CoA, from pantothenic acid and cysteine was also shown. This was confirmed to be due to the broad specificity for the nucleotide in the phosphorylation of pantothenic acid and its restriction in the coupling of 4'-phosphopantetheine with ATP. 3'-Dephospho-CoA was obtained by treating the reaction mixture which had accumulated CoA with 3'-nucleotidase of *Bacillus subtilis* IFO 3032.

Cell extract of *Brevibacterium ammoniagenes* IFO 12071 contained all the enzymes necessary for the operation of Brown's pathway for the biosynthesis of CoA. Pantothenoylcysteine decarboxylase, an enzyme in Novelli's pathway, did not detected. These observations led to the conclusion that Brown's pathway operates in this bacterium. It was considered that Brown's pathway also operates in a wide variety of microorganisms.

Pantothenate kinase, the enzyme catalyzing the first step in CoA biosynthesis, has been purified as a homogeneous protein from *Brevibacterium ammoniagenes* IFO 12071. The enzyme activity was inhibited by CoA and its biosynthetic intermediates. In many microorganisms tested, as well as *Brevibacterium ammoniagenes* IFO 12071, CoA inhibited the phosphorylation of pantothenic acid, and resulted in a decrease of CoA production. These results led to the suggestion that a feedback inhibition of pantothenate kinase by CoA may be involved in regulating the intracellular CoA level as a general regulation mechanism. On the contrary, in all the CoA producing strains, only little inhibition by CoA was observed in other reactions involved in CoA biosynthesis, and CoA production from 4'-phosphopantothenic acid by *Brevibacterium ammoniagenes* IFO 12071 was not repressed even in the presence of 4 mM of CoA. It was concluded that the higher accumulation of CoA from 4'-phosphopantothenic acid may be attributed to this less-sensitiveness of the enzymes.

The removal of permeability barriers of the cells by addition of surfactants was confirmed by leakage of the enzymes of CoA biosynthesis from the cells, and it was shown that CoA synthesis occurred partly extracellularly. All the CoA producing strains had considerably low degrading activities against CoA and AMP. This seemed to be one of the important features necessary for the

production of CoA.

On the basis of these results, a new process has been established for the production of CoA. Pantothenic acid, cysteine, and AMP when added to cultures of *Brevibacterium ammoniagenes* IFO 12071 gave CoA in a high yield. The product was obtained by using Duolite S-30, charcoal, and Dowex 1 x 2. The process has been shown to be adaptable for preparing large amounts of highly pure CoA, because it is simple, rapid, and compact, and requires no special equipment. Similarly, 4'-phosphopantothenic acid and 4'-phosphopantetheine were readily obtained in good yields.

Several microorganisms isolated from soil were found to grow in the medium containing pantothenyl alcohol as a sole carbon and nitrogen source. The results of the investigation of the degradative metabolism of this compound demonstrated that there are two different inducible pathways. In the pathway operating in strain 1041, pantothenyl alcohol was first hydrolyzed to pantoic acid and 3-aminopropanol, which was then followed by oxidation to β-alanine. In strain 1091, pantothenyl alcohol was first oxidized to pantothenic acid which was then hydrolyzed to pantoic acid and β-alanine.

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